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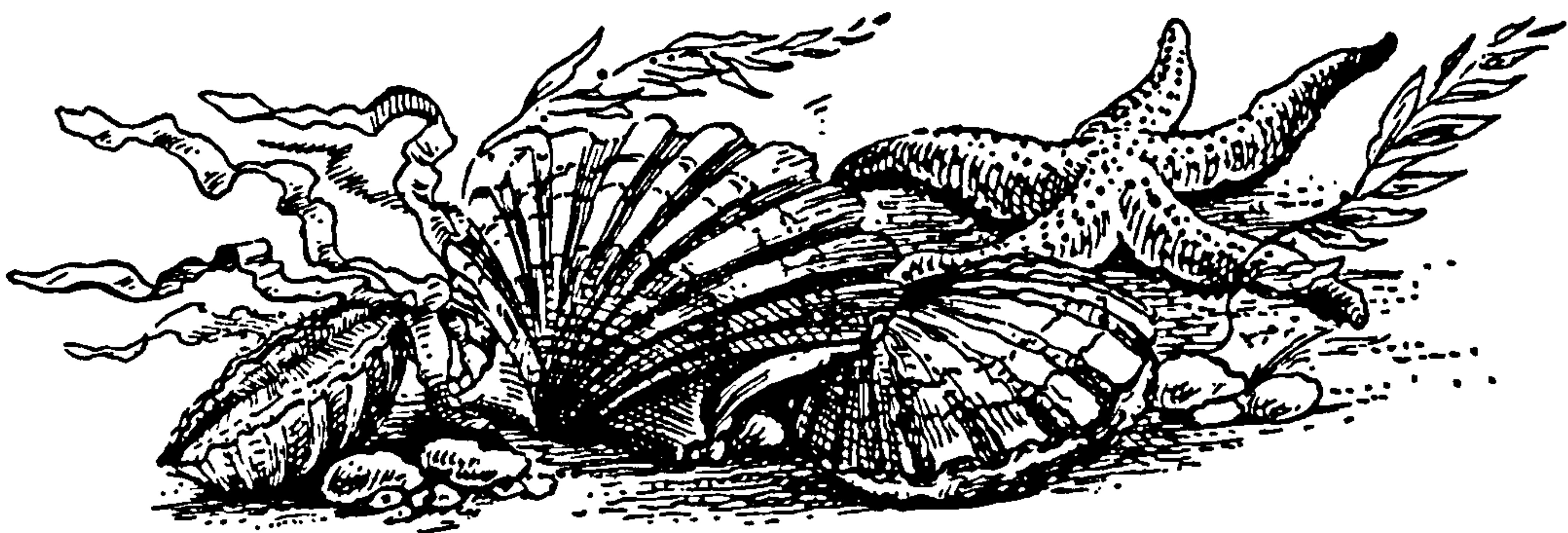
Post-harvest physiology of the scallop *Pecten maximus* (L.)

A Thesis submitted for the degree of Doctor of Philosophy
in the Faculty of Science at the University of Glasgow

by

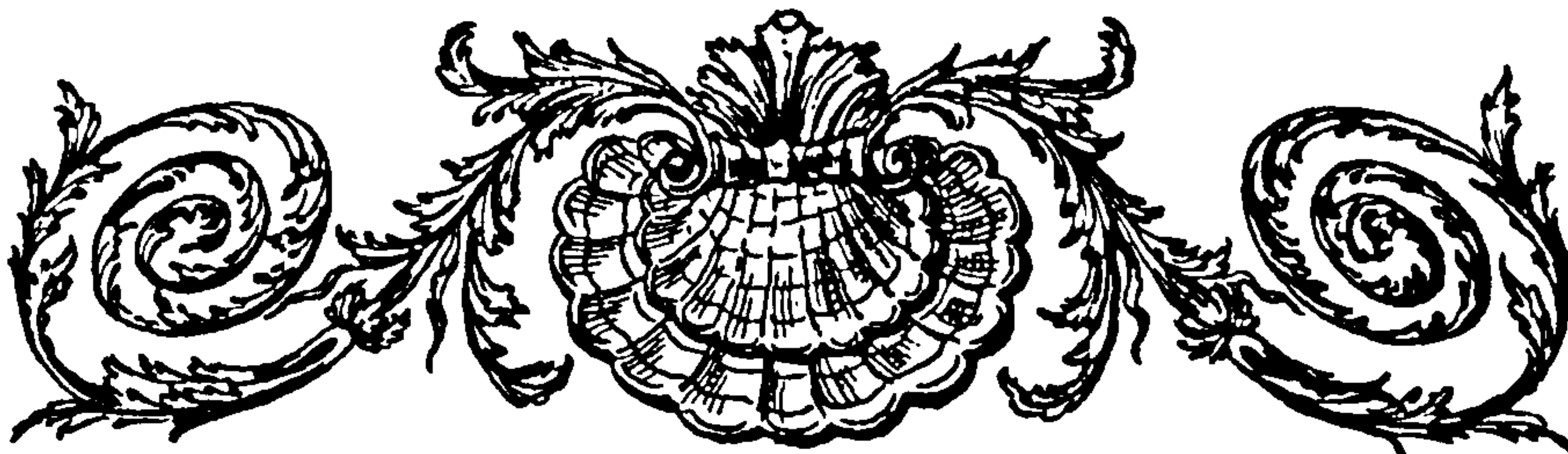
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Department of Zoology
October, 1993





This thesis is dedicated to the *Tyrannosaurus rex* in Kelvingrove Museum and Art gallery, who started it all, and to Dora, Lloyd, Karen and Alexis who helped me to finish it.



ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr. Alan C. Taylor and Dr. Peter Spencer Davies, for their guidance, advice and patience throughout the period of this work. Thanks are also due to them for enabling me to pursue most of my own ideas and for the experience of attending two International Pectinid Workshops. I am grateful to Professors R.S. Phillips and G.H. Coombs for making available the facilities of the Department of Zoology during the four years of this study.

Without the financial support of the Highlands and Islands Development Board (now Highlands and Islands Enterprise) and The Worshipful Company of Fishmongers this work would not have been carried out. I am indebted to these organisations and hope that the results of my work will be of benefit to the scallop industry. The Sea Fish Industry Authority marine farming unit at Ardtoe have been of considerable help since the beginning of the project and, despite their conviction that I was running a seafood restaurant in Glasgow, I would like to assure them that the scallops they provided died in the cause of science (mostly). I am grateful to the director, Malcolm Gillespie, for providing facilities and equipment during my two sabbaticals at Ardtoe, and particularly to Dr. Nick Lake for encouragement and assistance. Thanks are also due to John Macmillan, Mark Learmouth and Ian MacPherson. Mr. David Hardy was a mine of information on scallop diving and aquaculture and provided additional specimens of *Pecten maximus* when the restaurant was busy.

Within the University, workers in the Department of Microbiology and Laboratory for Biochemical Parasitology have also been of assistance. In particular Dr. Harry Birkbeck, Dr. Carol Mackie and Dr. Pauline Sinclair in Microbiology and Dr. Barbara Lockwood and Mr. David Laugland in Biochemical Parasitology. Ms. Margaret Mullen and Mrs. Maureen Gardner in the electron microscopy unit assisted with microscope use and photographic production.

In the Department of Zoology, Mr. Peter Rickus has produced many photographs and slides for me, often at short notice, and Dr. Willie Fowler was both enthusiastic and helpful during the histochemistry experiments. Cathy McLagan, Liz Denton and June Freel have been invaluable on many occasions and will doubtless be glad to see the back of me.

Finally, I would like to thank my parents for enabling me to become a zoologist, Karen for taking second place to an invertebrate and Kate, Keith, Jason and Kenny for dispensing beer and sanity when most needed.

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Duncan, P.F., Spicer, J.I., Taylor, A.C., Davies, P. Spencer

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Abstract

The scallop *Pecten maximus* (L.) forms the basis of an important fishery on the west coast of Scotland and, in recent years, has been successfully developed as an aquaculture species. Much of the production is processed with the edible portions, the adductor muscle and gonad, being sold as a fresh or frozen product. The largest markets for scallops lie in continental Europe, principally France, Spain and Belgium, and in these countries a premium is paid for large, in-shell scallops between 10 and 15cm shell height. The animals must, however, arrive at market in live condition and show little, or no, sign of shell valve gape, otherwise the consignment may be rejected.

Live shellfish are transported in air, i.e. emersed, due to the uneconomic cost of water carriage and, since scallops survive poorly in air, this physiological constraint is a major obstacle to the full development of the European market. The results of a survey, carried out to determine the nature of the live scallop market, indicated that, with current packing and transit conditions, live scallop deliveries could only be guaranteed up to 1100km (approximately 60 hours transit time) from Scottish production centres. Detailed results of this survey are presented.

Experiments were carried out to identify the environmental factors influencing survival during emersion with the aim of suggesting modifications to present transit conditions in order to minimise physiological stress, thus improving scallop condition and survival. Results showed that increasing the emersion temperature from 5 to 20°C caused a reduction in maximum survival time of 62%. Similarly, reduction in relative humidity, from 95 to 70% saturation, resulted in a 27% reduction in maximum survival time. Scallops exposed to a fine sea-water mist showed a 13% improvement in survival and the survival of scallops tested in December was 67% better than those tested in July. This seasonal effect appeared to be correlated with tissue glycogen concentrations which decreased by 29% over the same period. Maximum survival of any emersed scallop was 166 hours, although the animals were not in an edible condition after this time.

Experimental factors which were found not to influence survival directly were packing orientation, enforced shell-valve closure and hyperoxic conditions (~100% oxygen).

These results implicated aspects of energy metabolism as the primary causes of scallop death during emersion and therefore this area was investigated in more detail. Further work showed that heart rate had a positive linear relationship with temperature until ~19°C. Thereafter, heart rate became irregular and uncoordinated, suggesting that poorer survival at

high temperature is caused by loss of physiological integration and difficulty in meeting increased metabolic demands.

The oxygen consumption rate of *Pecten maximus* in air is reduced to only 50% of the aquatic rate within 8 hours of emersion. This decline in respiration rate continued and was typically between 25 & 30% of the aquatic oxygen consumption rate after 60 hours in air. In addition, heart rate, haemolymph PO_2 , haemolymph pH and mantle cavity fluid PO_2 and pH all decreased during emersion, implying that respiratory and circulatory systems were deteriorating.

Although oxygen consumption was found to decrease upon emersion, an equivalent reduction in metabolism was not apparent, since rapid valve adduction occurred and the accumulation of ammonia in the haemolymph and mantle-cavity fluid, derived from nitrogen metabolism, was constant over a 48 hour exposure. This implied that the energy for metabolism was not being supplied exclusively via aerobic pathways and, hence, anaerobic metabolism was probably employed to some degree. Use of anaerobic pathways by a sublittoral species, such as *Pecten maximus*, is unlikely to supply sufficient energy to sustain an active metabolism for an extended period and the animal may accumulate harmful waste products for which it has little tolerance. Without recourse to usable oxygen, to boost energy production and re-metabolize the anaerobic waste products, the physiological status of the scallop will deteriorate and death will result.

Analysis of muscle tissue using HPLC indicated a reduced energy state after 12 hours in air and also the accumulation of acids derived from anaerobic metabolism. These factors were considered to be the cause of the observed reduction in valve adduction frequency during emersion (by 94% within 6 hours), and also the gradual increase in valve gape up to 12 hours. As the emersion period progressed, the index of free energy (adenylate energy charge) continued to decrease and reached an irreversible, and fatal, level within 48 hours. Acid accumulation also continued and the presence of propionate was indicative of severe hypoxia. Although considered to be directly responsible for adductor muscle failure, these factors are also likely to be indicative of whole animal status and may therefore contribute to deterioration in other tissues and thus affect overall survival.

The problems of oxygen supply during emersion result initially from loss of gill support as water drains from the mantle cavity, or is expelled by valve adduction. As the gill filaments clump together the area for gas exchange is substantially reduced causing the initial rapid decrease in oxygen uptake and internal oxygen concentration. These parameters continue to decline as the efficiency of gas exchange by the gill is affected by the accumulation of ammonia in the haemolymph and in the remaining water surrounding the gills. After 48 hours in air ammonia concentrations in these fluids were found to have increased by factors of 25 and 50

respectively. Examination of gill tissue from emersed scallops, using both scanning (S.E.M.) and transmission (T.E.M.) electron microscopy, showed substantial damage to the epithelial layer and to the functional structure of the organs.

Gill tissue damage was also caused by the activity of bacteria which proliferate rapidly in mantle-cavity fluid and on the tissue surfaces of emersed scallops. Bacterial numbers increased by 10^7 fold within 7 days and were found to produce enzymes which broke down gill tissue and inhibited ciliary activity. Their effect on overall survival was demonstrated by application of antibiotic compounds which reduced bacterial numbers for up to 5 days and resulted in an improvement of up to 15% in the survival time of emersed scallops. Bacteria were isolated from emersed scallops and identified to genus level where possible. The results were typical of the microbial flora of marine bivalves and included the following taxa; *Vibrio/Aeromonas*, *Pseudomonas/Alcaligenes*, *Flavobacterium*, *Micrococcus*, *Agrobacterium*, *Cytophaga*, *Acinetobacter*, *Moraxella* and Enterobacteriaceae.

Based on the experimental results of this study, a number of potential improvements to current post-harvest handling and transportation of *Pecten maximus* are suggested.

1.1. Production of commercially important scallop species in Scotland

Worldwide there are more than 380 species and sub-species of scallop within the families Pectinidae and Propeamussiidae, inhabiting polar, temperate and tropical waters (Rombouts, 1991). Twelve species of scallop are found around the coast of Scotland (Tebble, 1966; Rombouts, 1991). Of these, only two are commercially important, *Pecten maximus* (Linnaeus, 1758) (the great scallop) and *Aequipecten* (= *Chlamys*) *opercularis* (Linnaeus, 1758) (the queen scallop), although the former has a much greater value on account of its higher meat yield and relatively lower abundance. This research project is concerned solely with *Pecten maximus* and the word 'scallop' is used with reference to this species, unless otherwise stated.

Pecten maximus has a wide coastal distribution on the Atlantic coast of Europe, generally ranging from northern Norway to the Iberian peninsula (Tebble, 1966), although it has been reported off west Africa, the Azores, the Canary Islands and Madeira (Mason, 1983). It prefers a clean sand or fine gravel substrate in depths from the low water mark to 183m (Forbes & Hanley, 1853), but is most common between 18 and 46m (Mason, 1983).

1.2. The development of the scallop fishery

Commercial exploitation of scallops in Scotland began on a small scale in the latter part of the 19th century when scallops, mainly *Aequipecten opercularis*, were used as bait for the important line fishery, but this use declined during the 1920's as more efficient net capture systems were developed. Shortly after this time a small dredge fishery for *Pecten maximus* developed in the Firth of Clyde which continued throughout the 1940's and 50's. All of this catch, approximately 100 tonnes/annum, was sent live to Billingsgate, London, packed into sacks with the right valve down in order to retain water (Mason, 1983). This market was seasonal, however, partly on account of poor meat and roe condition following spawning, but mainly due to problems of live transit during the warmer summer months. The dredge fishery therefore remained stable until the early 1960's when the use of refrigerated transport overcame the delivery problems to southern Britain, and the development of new machinery enabled more efficient production of processed shellfish for the large European market. This market, comprising predominantly of France, Belgium and Spain, had an almost insatiable demand for a 'roe-on' product, i.e. adductor muscles and gonad separated from the viscera, which could be supplied either fresh or frozen. The influence of this market development

resulted in Scottish scallop landings increasing to more than 4400 tonnes in 1969 which, despite occasional fluctuations, continued until a peak of 6532 tonnes was reached in 1982. Since then landings at Scottish ports have declined, although the value of the catch has increased (Fig. 1.1). At present, scallops represent the most valuable 'shellfish' species, after *Nephrops norvegicus*, with a total value of almost £7M. in 1991 (S.O.A.F.D., pers. comm.).

The high value of *Pecten maximus* over the past decade has resulted in a greater fishing effort by traditional dredging methods. Despite this, official statistics indicate that the catch per unit effort has fallen by 42% over the same period. This has led to the development of new production methods and the introduction of pectinid aquaculture which, by providing a different quality product, have been able to capitalise on the recent upsurge in demand for fresh, rather than processed, seafood. As a consequence, these new industries have not necessarily been in direct competition with the traditional fishery.

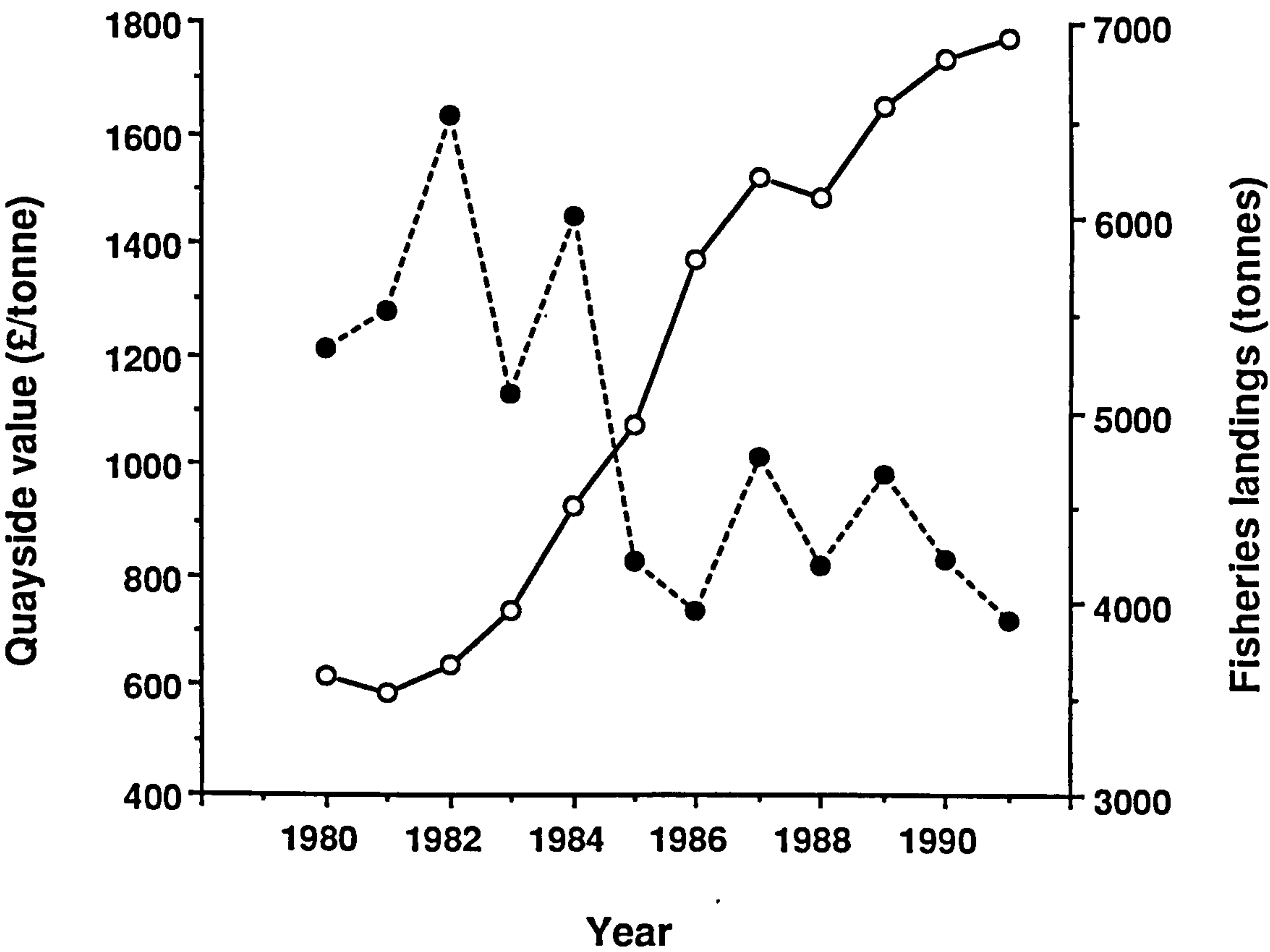
1.3. Present fishery methods

Dredging

The most common method of scallop fishing involves the use of vessels originally designed for white fish trawling, which require little or no modification to tow scallop dredges. This has enabled many boats, previously involved in north and east coast demersal trawling, to move into scallop fishing when quotas or stocks are exhausted. Ansell *et al.* (1991) estimate that, in recent years, 120 boats were actively involved in scallop dredging, primarily on the west coast. A detailed description of dredge construction and use is given by Mason (1983). Between 10 and 20 dredges may be deployed at one time and typical catches on a productive ground may average 50 scallops/dredge/hour. Scallops are stored in sacks, 10-15 dozen per bag depending on size, for up to 3 days prior to landing. Ice may be used in packing during the summer months.

Unfortunately, dredge fishing frequently results in the mantle cavity of the scallop being filled with grit and sand, and chipped or broken shells often cause damage to the mantle tissue itself. Consequently, the majority of dredged scallops are unsuitable for the live market on account of contamination and poor survival caused by dredging and handling stress. A number of trawler companies have attempted live transport of dredged scallops, following a 3-4 day recovery and cleaning period in storage tanks. However, this practice is exceptional and since mortalities are high, it becomes economically viable only if the value of live scallops far exceeds the value of

Fig. 1.1 Changes in the quayside value and landings of *Pecten maximus* at Scottish fishing ports between 1980 and 1991. Open symbols represent the average quayside value in £/tonne (in-shell weight), closed symbols represent the total landings by U.K. vessels. Data obtained from The Scottish Office Agriculture and Fisheries Department (1992).



processed scallops. Dredged scallops are, therefore, almost exclusively sent for processing, with boats landing close to the processor offering the optimum price. The processed scallop, as a fresh, frozen, roe-on or roe-off product, constitutes approximately 90% of the total scallop market (de Franssu, 1990).

Aquaculture

Pectinid aquaculture began in Scotland in the early 1980's, employing the successful techniques developed by the Japanese (Ventilla, 1982), although experimental spat collection and ongrowing trials, by organisations such as the White Fish Authority (Sea Fish Industry Authority) and D.A.F.S. (now S.O.A.F.D.), were carried out throughout the 1970's. In addition, university based research contributed to a better understanding of the biology of potential culture species (Soemodihardjo, 1974; Paul, 1978; Taylor & Venn, 1979; Burnell, 1983; Mackie, 1986). Fundamental knowledge of adult distribution, reproductive cycles, larval biology, growth rates and predation was essential since viable culture depended on reliable and efficient capture of wild larvae using artificial collectors (Brand *et al.*, 1980). Hatchery production of scallop larvae (spat) has been investigated for a number of years (Comely, 1972; Gruffydd & Beaumont, 1972; Beaumont *et al.*, 1982), but large scale, commercial production of larvae is still some years distant. Ongrowing of juvenile scallops is based on suspended culture, either in lantern nets (Paul *et al.*, 1981) or by 'ear' hanging (Paul, 1988). Suspension in the water column, with relatively higher temperatures and plankton concentrations, results in a faster growth rate and a much higher meat yield in comparison with epibenthic scallops (Gillespie, 1983; Paul, 1988; Duncan, 1989). However, suspended culture, particularly 'ear' hanging, also results in excessive marine fouling of the shell valves by algae, serpulid worms, barnacles or ascidians, depending on the culture area. As a result, many of the scallops produced are unsuitable for the live market and consequently processing is the preferred option. External fouling can be removed by relaying scallops on suitable substrata which results in suffocation or predation of the fouling organisms when the scallop recesses. Many growers are discouraged from this practice, however, due to the requirement for diving or the use of dredging equipment to recover the animals.

The development of SCUBA over recent years has encouraged many growers, often ex-commercial divers, to expand the fouling-removal procedure into a viable culture method in itself. This technique, known as ranching, has proven to be both viable and popular, particularly due to the increasing difficulty of obtaining suitable longline sites for suspended culture. Ranching probably originated from the practice of scallop divers seeding known and suitable areas with undersized stock, thus allowing the animals to grow and be harvested later (Hardy, 1981). With the advent of

culture techniques it then became possible to collect natural spat, maintain them in suspended culture for 2 years, before releasing juvenile scallops onto the seabed. Thus, the latter part of the culture cycle required little capital equipment or maintenance until final harvesting. Despite a slightly longer overall growth period in comparison with exclusively suspended culture systems, the problems of predation (Lake *et al.*, 1987) and legal tenure over the stock (S.F.I.A., 1988) ranching has been successful, particularly under a crofting or cooperative farming structure, or when combined with other sources of income.

Scallop culture in general would appear to be commercially viable only if it is carried out in conjunction with other activities, since unreliable spat collection, high initial capital outlay and long financial lead time (i.e. time to first sales), due to the long growth period, makes large scale operations impractical. A detailed view of all aspects of practical pectinid culture in Scotland is given by Hardy (1991). Official statistics estimated that in 1990/91, 290 shellfish farms were registered, with 229 considered active (McKay, 1991). Of this total approximately 15% were involved in pectinid culture (H.I.D.B., 1989), although the majority concentrated on suspended culture of *Aequipecten opercularis*. Total farmed production of *Pecten maximus* in 1990 was recorded as 105,500 scallops (12.7 tonnes) per annum (McKay, 1991), although this figure was probably a substantial under-estimate judging from personal enquiries. Official statistics for 1992 have shown an increase in production to approximately 60 tonnes per annum, although this is also considered to be underestimated by as much as 250% (N.C.H. Lake, pers. comm.). The majority of farming operations use suspended culture techniques, at least for the initial on-growing stage, although there is now a growing tendency to transfer to on-bottom culture after two years of age. This practice will probably become the standard, as granting of stock-protection rights (several orders) become easier to obtain.

Commercial scallop diving

The remaining source of scallop production, which represents a significant, but largely unregistered, contribution to total figures comes from the harvesting of wild stock by SCUBA diving operations. A full account of this activity is described by Hardy (1981). Although limited to some extent by depth, diving has the advantage of being able to exploit stocks which are unavailable to commercial dredges. Therefore the two fisheries do not compete and the diver is able to land large, unfouled scallops which are predominantly supplied to the live, in-shell market. In addition, recent concern about the effect of sustained dredging on other benthic fauna, including scallops which evade capture, make diving operations less damaging to the marine environment, provided that sustainable fishing practices are observed. The increasing popularity of sport diving and direct

entry into the most lucrative area of the scallop market has resulted in a relatively large number of commercial operations being established on the west coast of Scotland in recent years. Information obtained during this study estimates that 50-60 diver fishermen operate between Islay and Lewis, including the mainland, and collect up to 90,000 scallops each per year. This gives an overall production of about 750 tonnes per annum (assuming 150g/shell) worth approximately £3M (1992 price).

2.1. The live scallop market

The total world production of all commercial pectinid species in 1988 was estimated at nearly 900,000 tonnes (in-shell weight) (de Franssu, 1990). Japanese aquaculture and the USA fishery accounted for 38.4 and 27% of the total, while Great Britain produced 14,700 tonnes (1.65%). The international pectinid trade, by product form, which covers only imported scallops, is shown in Table 2.1.

Table 2.1 The world scallop market by product. Figures relate to all commercial species.
Modified from de Franssu (1990). Value converted at \$1.5 (U.S.) to the £.

Product	Quantity (tonnes)	Value (£M)	Main Markets
Fresh (live), in-shell	3000	13.3	France, Spain, Italy, USA
Fresh, shucked	2000	20	USA, France
Frozen meat/roe	30000	166.6	USA, France, Belgium
Total (equivalent live wt.)	300000	200	/

Accurate figures for the proportion of the Scottish production sold live, in-shell are difficult to obtain, since live and fresh scallops of all species are combined in import/export data. However, data from a number of sources (H.I.D.B., 1988; S.O.A.F.D., 1988; 1990; de Franssu, 1990; Bamfield, 1991; This study) indicate that approximately 10% of the total *Pecten maximus* production is sold live. Live, in-shell scallops are only marketed relatively close to the centres of production on account of the well recorded inability of these bivalves to survive long periods out of water. All molluscan shellfish are transported and sold out of water due to the additional and uneconomic cost of water shipment. The poor survival ability of scallops has generally been explained by dehydration since the shell valves do not seal together, particularly at the anterior and posterior dorsal margins, thus allowing retained water to seep out during transit (Mason, 1983; de Franssu, 1990).

Recent statistics (Monfort, 1992), indicate that scallop consumption in France is increasing, particularly of the fresh Scottish product, a significant proportion of which is imported live (Table 2.1). France has, in addition to most other EEC countries, a demand for scallops which far exceeds domestic supply. For example, Belgium imports 81% of total consumption (60% from U.K) and France 52% (76% from U.K) (Bamfield, 1991). Britain is unusual in having a particularly under-

developed domestic market, and is consequently a net exporter. It would appear, therefore, that Scotland is in a good position to take advantage of continental markets if production of suitable live scallops could be increased and improvements in condition and survival can be attained.

2.2. Present transportation methods for live scallops

A prerequisite for the improvement of survival of live scallops whilst in transit is a knowledge of current market practices. During the present study, personal interviews and questionnaires were employed to obtain information from producers, distributors and retailers concerning the species dealt with, how they are obtained and handled post-harvest and whether they recognised any problems of scallop transport which might limit development of the market.

The following questionnaire was devised and employed where personal communication was not possible or where large numbers of people could be more efficiently reached through an intermediate contact. Where applicable, alternative questions were included depending on the individuals being targeted.

Questionnaire:

(Producer format)

- 1) Which species do you catch / grow?
- 2) How and where they are caught / grown?
- 3) How are they handled onboard or on shore after harvesting?
- 4) What is the average and maximum exposure to air between;
 landing on boat and landing on shore?
 landing on shore and loading onto transit vehicle?
 transit time and distance to market (longest, shortest average)?
- 5) Are there any special conditions used by you or the hauliers to keep the scallops cool or damp?
- 6) Do you sell to processors, live trade, other (specify)
- 7) Who are they sold to; by you, ultimately?
- 8) Selling frequency?
- 9) How does buyer assess acceptability of a live delivery?
- 10) Do you take special measures to ensure acceptable delivery? e.g. packaging, maximum delivery distances or times, other (specify)

Questionnaire:

(Haulier format)

- 1) Which of the following shellfish do you transport live?

Scallops
Queen scallops
Princess scallops
- 2) By which methods are they transported?
- 3) How are they transported within these vehicles?

Vivier tanks (water temperature?)
Cascade system (water temperature?)
Ice
Damp packaging
Chilled (temperature?)
Boxed (material type?)
Bagged (material type?)
- 4) How far are they transported?
- 5) How long does this take?
- 6) Are they expected to arrive live?
- 7) What is the maximum range to ensure live delivery, by which transport method?
- 8) Are there any special conditions used to extend life?
- 9) Are you responsible for delivery condition?
- 10) Who do you deliver to, market, retailer, restaurant, other (specify)?
- 11) What quantities do you deliver?
- 12) How often do you deliver and to which destinations?

The survey information was obtained mainly via telephone or personal interviews, although a total of 15 questionnaires were distributed to remote producers and hauliers of which 8 were completed and returned. Due to the commercial nature of the survey some interviewees/respondants were unwilling to answer questions as fully as possible. Other questions, particularly within questionnaires, were misunderstood or answered rather vaguely. Consequently, the sample sizes presented in Table 2.2 may not represent the total obtained for each question. Full details of the survey are described in Appendix 1.

Table 2.2 Number of individuals, or representatives, from specific groups involved in the production and marketing of live scallops who contributed information to the present survey. *Producers include all forms of production except dredging.

Group designation	Fishermen	*Producers	Hauliers	Processors	Fish merchants
Number of contributors	2	10	7	4	7

The findings indicated that the live market is concerned solely with *Pecten maximus*, the majority of which are diver caught, but with a small contribution from cleaned, cultured scallops. Although specific fisheries legislation applies only to wild caught stock, market requirements also specify a minimum scallop size. For this reason the scallop should be greater than 110mm shell length to be sold live, whether from diver or aquaculture production, and preferably between 120 and 150mm. The market for *Aequipecten opercularis* is entirely processed, with the possible exception of juvenile animals which may be sold as 'princess' scallops. However, no producer questioned dealt with this product and the two largest growers of princesses known to me ceased trading 2-3 years ago. This market may therefore be considered insignificant.

Most producers involved in diving for large scallops combine this operation with spat collection, ongrowing and ranching. Others were concerned solely with either diving or aquaculture production. After harvesting, the immediate on-board treatment by both types of producer involved packing into sacks and storing the scallops on the deck. This practice resulted in a maximum emersion of eight hours before the scallops were landed on shore. Most producers specified that during this period the scallops were either kept under moist covers or regularly hosed with sea water. Divers transferred each day's catch to keepnets which were stored on the seabed until delivery was required or sufficient scallops had been caught to satisfy a particular order. Aquaculture producers, who maintained their stock in known localities and quantities, harvested the required number of scallops for each order and had pre-arranged transport awaiting delivery at the quayside. Depending on the size of the operation, deliveries varied from twice weekly to annually, at the peak demand period (December/January).

Most producers employ specialist fish/shellfish hauliers for transit, although some use their own transport, usually in conjunction with a continental parent company. Packing is relatively

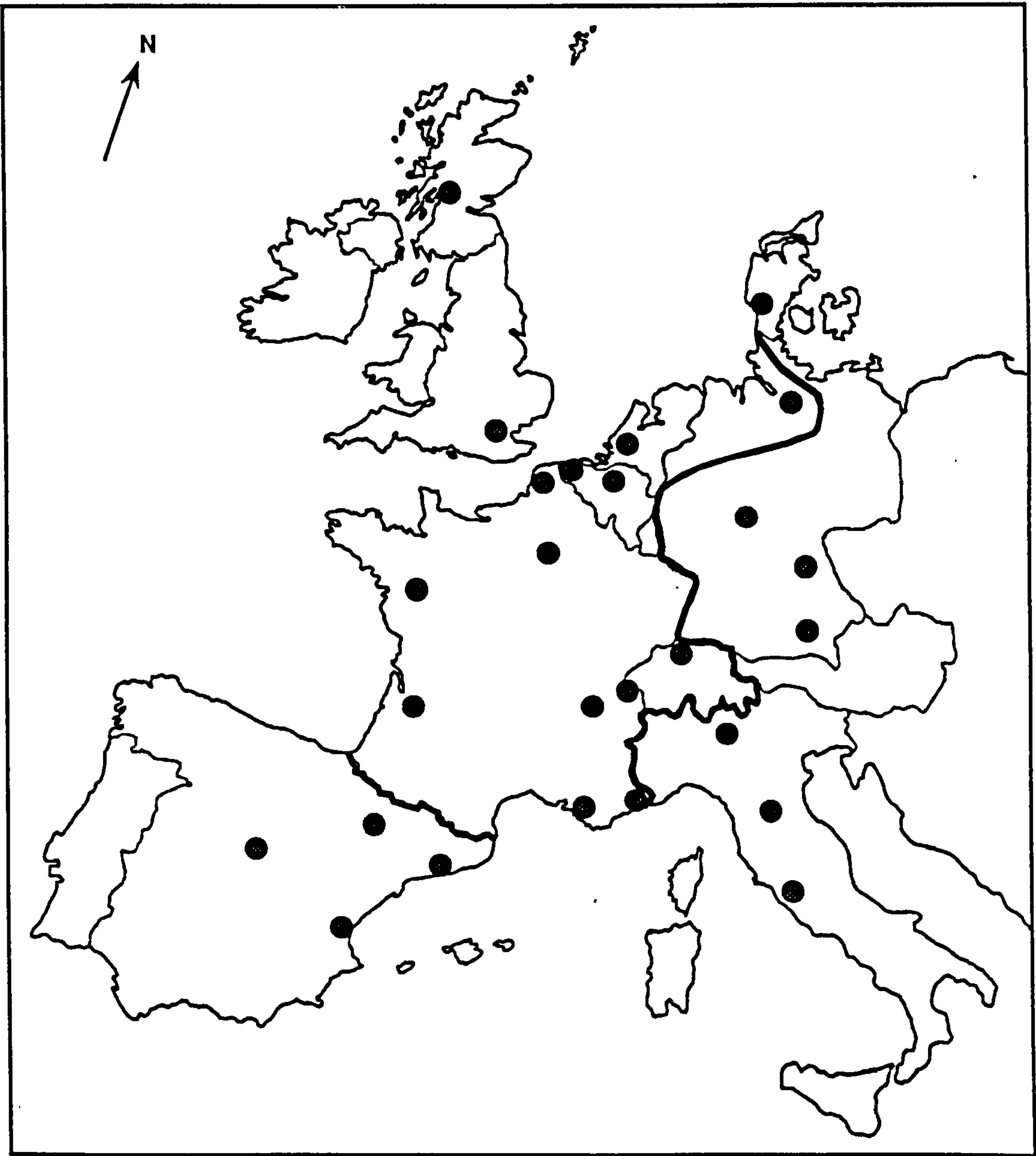
standard with polypropylene sacks or 10kg polystyrene boxes being used. The latter are more common for longer transport distances. Small growers who operate independently usually sell their produce locally or within Britain and transit time is between 1 and 12 hours. Local deliveries require no special treatment. Deliveries to England, when transit time may exceed 10 hours, require refrigerated transport at 4°C and additional packing with ice is often employed.

Hauliers who specialise in live-scallop transport, often large producers themselves or individuals who accumulate the catches of local divers, are generally involved in road haulage shipments to continental markets where the best prices are obtained. These shipments, which are usually weekly, deliver predominantly to France, Belgium, Holland and Germany and take between 24 and 60 hours for delivery. In these cases scallops are packed tightly with the right valve downwards to avoid water loss (and possibly adduction and gape). Polystyrene boxes or sacks are used for packing, in conjunction with chilled transit vehicles.

Live, in-shell scallops are required to arrive in good condition and acceptance of the product depends on visual appearance, mantle response and the absence of shell gape and odour. It was frequently reported that the producer/buyer relationship was also of significant importance for product acceptance. Continental parent companies and long established customers trust the producer as to the time of harvest, packing treatment and transportation condition. Consequently, dead or poor quality scallops are rare. Only exceptional conditions, or entry into a new, unknown market, are likely to result in consignment rejection. Scallop producers are likely to seek new customers around existing markets, where they can guarantee acceptable delivery, and avoid developing new areas which are deemed marginal due to distance or time. This conservative, though sensible, approach means that the majority of producers and hauliers do not recognise an absolute maximum distance or time for guaranteed delivery of live scallops since they always work within a well tested timetable. The maximum road delivery distance reported during this survey was approximately 700 miles (1120 km) and the maximum delivery time was 60 hours. The latter figure appears to be close to an absolute maximum and, when combined with data obtained from haulage companies, it is possible to produce a diagrammatic representation of the theoretical range for live-scallop transport by present methods (Fig 2.1).

Destinations outwith this 60 hour range effectively represent a closed market to conventional road transit and additional evidence for this comes from the reported transit practices to countries such as Spain and Portugal, both of which lie outside the suggested range. Both of the respondents who regularly ship to these markets used air freighting to guarantee the arrival of scallops in good condition. This method represents a significant saving in time since the maximum air transit time to

Fig. 2.1 Approximate range limit for road transit of live *Pecten maximus* within Europe. Red boundary indicates the 60 hour transit distance from the west coast of Scotland which survey results estimate to be the maximum transit time in order to ensure acceptable delivery. Transit times relate to the most appropriate ferry route and are based on haulage company data. Political boundaries are indicated by full lines, main fish marketing centres are indicated by hatched circles. Approximate scale: 1cm=180km.



Spanish markets was reported as 32 hours. Air transport is, however, significantly more expensive than road haulage and is commercially viable only at peak demand periods or when sending scallops to 'make up' the weight of a more valuable shipment such as *Homarus gammarus* or *Nephrops norvegicus*. Air transport also requires the use of leak resistant packaging and therefore scallops are delivered exclusively in polystyrene boxes with sealed ice packs or damp packing.

2.3. Conclusions

Maximum transit time using present packing and transit methods appears to be approximately 60 hours in order to ensure acceptable delivery condition. Potential markets in southern Europe, and some parts of north-western Europe, are therefore largely excluded given such limitations, unless circumstances prevail which justify faster, although more expensive, modes of transport. Current market trends indicate an increasing demand for live scallops in Europe which is unlikely to be met by traditional fishing methods, when present fishing effort and landings data are considered.

Scotland is in a unique position to dominate this potential market since it presently has the largest stocks, distributed over a large fjordic coastline. This situation could allow development of aquaculture and diving operations sufficient to supply a large proportion of European demand. However, the most valuable sector of the scallop market, the live trade, could remain under-developed because of problems associated with transport to more distant markets. Although the live scallop price remains relatively high, it would be required to increase substantially to financially justify the cost of single species air freight.

Alternatively, if research could determine the important factors which cause deterioration and death of *Pecten maximus* during transit, then modification of environmental or packing conditions may lead to improvement in survival, thus allowing greater development of distant markets or product improvement for existing points of sale. The aim of this project, therefore, was to identify the reasons behind the well recorded inability of *Pecten maximus* to survive extended emersion. Desiccation has been proposed as the principal cause of scallop death, presumably due to its important influence on the survival of littoral species during tidal emersions. Conditions during transit, however, are dissimilar to those encountered on the exposed shore and it is likely that other environmental parameters are equally important. Identification of these factors, and determination of the physiological changes which they cause would enable us to suggest what causes post-harvest death of scallops. Given that *Pecten maximus* lives entirely below the lowest tidal level, i.e. it is a sub-littoral species, it is unlikely to have specialised adaptation to emersion. This will limit the

means of improving survival to manipulation of the transit conditions themselves, in order to minimise the rate or extent of physiological deterioration. The initial requirement of this study, therefore, was the identification of controllable environmental factors which have a direct influence on survival via their effects on physiological systems.

Chapter 3 Assessment of factors affecting scallop survival during emersion

Introduction

Realization of the market potential for live *Pecten maximus* depends largely upon improvement of transportation methods which will allow the scallops to survive longer and arrive at market in better condition. Clearly, before any attempt can be made to improve the transit system, the factors which affect survival and condition must be determined. As mentioned in the previous chapter, live scallops are transported out of water, i.e. emersed, and therefore, while little published work has related specifically to the physiological changes associated with shellfish transport, it seems likely that factors implicated with physiological stress and survival limitation in other emersed bivalves will be relevant in this context.

Most previous studies on emersion in bivalves have concentrated on the effects of short-term tidal exposures on littoral species (Newell, 1973; Bayne *et al.*, 1976; Widdows *et al.*, 1979; McMahon, 1988). Perhaps not surprisingly there has been little research into the effects of aerial exposure on sub-littoral species, such as *Pecten maximus*, since under normal conditions this environmental situation would not arise. During transportation, scallops would therefore encounter environmental conditions to which they have no specific adaptation. It follows that the stress caused by these conditions is likely to be greater than in intertidal species. The factors most likely to lead to stress and mortality during emersion will be;

1. Temperature change
2. Low humidity and the effects of desiccation
3. Oxygen availability
4. Accumulation of metabolic and excretory products
5. Condition and post-harvest treatment of the scallop

Bayne (1975), defined stress as a measurable alteration of the physiological steady state caused by an environmental change, leaving the animal more vulnerable to further environmental change. The aim of the initial work, therefore, was to assess the importance of these environmental factors by measurement and comparison of survival rates during emersion. Those factors which were found to affect survival could then be examined in greater detail by measurement of the physiological changes which they caused with a view to perhaps controlling their effects and hence improving condition and reducing mortality.

Temperature change can affect an organism in a number of ways. For example, by changing the structure of biochemical molecules, through its action on non-covalent or 'weak' chemical bonds, temperature fluctuations can alter chemical interactions between molecules and

affect the functional properties of protein-phospholipid membranes (Schmidt-Nielson, 1990). Additionally, variation in the kinetic energy of molecules, caused by temperature alteration, will be apparent as changes in the rate of chemical reactions (Hochachka & Somero, 1973) and hence, increased temperature tends to increase the metabolic rate of an animal. This will have the effect of making greater demands on the respiratory system by increasing the rate of oxygen consumption or forcing the adoption of anaerobic pathways if hypoxic conditions prevail. Furthermore, a higher metabolic rate will increase the production rate of excretory products, which must be dealt with if the animal is to avoid accumulation and subsequent toxic effects.

Desiccation is considered to be one of the main environmental stresses encountered by littoral animals and an ability to withstand or control water loss frequently determines survival in the intertidal zone (Morton *et al.*, 1957). Prevention of desiccation has also been recognised within the shellfish industry as being important during transit and, as a result, many commercial species of mollusc and crustacean are packed and transported with moist material such as damp tissue or seaweed. Desiccation may contribute to mortality by reducing respiratory efficiency, due to drying of respiratory surfaces, or by cellular based physiological changes resulting from an increase in the osmotic concentration of extra-cellular fluid caused by water loss.

Another significant problem which aquatic organisms have to overcome during aerial exposure is that of maintaining the oxygen supply to aerobically respiring tissues. This is not necessarily a problem of oxygen availability, as some high-littoral bivalve species can maintain aquatic levels of respiration during emersion by absorbing oxygen across moist tissue surfaces (Boyden, 1972a; Deaton, 1991). These species are exceptional, however, since the majority of bivalves cannot meet their total oxygen requirements in air due to inadequate gas uptake or delivery mechanisms and the danger of desiccation by exposing moist surfaces. Consequently, most intertidal bivalves close their shell valves tightly during emersion, preventing diffusion of oxygen to the gills, and resort to anaerobic energy production (Akberali & Trueman, 1985). Following reimmersion, and return to aerobic conditions the oxygen debt is paid off and both oxygen consumption and heart rate are increased. These responses are associated with oxidation or conversion of accumulated excretory products, recovery of phosphagen energy pools and the expulsion of waste metabolites such as ammonia (de Zwann, 1977; Ellington, 1983a). Physiological strategies such as the ability to use organic substrates as electron/proton acceptors (Akberali & Trueman, 1985) and the capacity to buffer the accumulated acidic end products in order to avoid pH imbalance (Crenshaw & Neff, 1969) are most developed in intertidal bivalves which encounter regular tidal emersion. It was anticipated that *Pecten maximus* as a subtidal bivalve would lack these physiological adaptations and, furthermore, during transit, the opportunity for aerobic recovery in water would not be available. The

survival of *Pecten maximus* in air may therefore be limited by an inability to utilise atmospheric oxygen.

The packing method employed for transportation of shellfish may also affect survival. Packing orientation of scallops has traditionally been with the right (convex) shell valve down. This was intended to retain water within the mantle cavity which was believed to provide both support for the gills and a reservoir for dissolved oxygen. Another widely used packing strategy is that of preventing shell adduction, which results in the rapid expulsion of water from the mantle cavity during transit. Tight packing of the scallops was thought to avoid this problem, but the practice could increase mortality by reducing air circulation, thus creating a stagnant, oxygen depleted layer in contact with the gills. If access to atmospheric oxygen is a limiting factor in the survival of scallops during emersion, either due to morphology or packaging method, then enhancing the available oxygen concentration may improve survival.

The emersed scallop also encounters the problem of accumulation of toxic metabolic end products. Marine bivalves are primarily ammonotelic, producing ammonia as the main nitrogenous waste compound. Under immersed conditions the toxicity of this substance is rapidly reduced by dilution as the urine is excreted into the surrounding water (Newell, 1964), or possibly directly via gill excretion. When emersed, however, toxic excretory products, including ammonia, can accumulate in the mantle cavity of bivalves (Bayne *et al.*, 1976; de Vooy & de Zwann, 1978), with potential implications for survival. Metabolic end-product accumulation may be particularly significant in deep, active tissues such as the adductor muscles since reduced oxygen penetration and a high work load results in rapid production of metabolites from anaerobic pathways (Gäde, 1983). Tolerance of these products is usually limited and under immersed conditions, the activity period is short with product breakdown occurring as the animal recovers (Ellington, 1983a). Without recourse to aerobic recovery, the emersed scallop in transit will continue to accumulate end products which could cause cell damage and lead to loss of physiological function. Removal of deleterious metabolites from bivalves, either from anaerobic or nitrogen metabolism, normally requires the animal to be immersed, but if emersed scallops could be provided with sufficient water to flush the mantle cavity or enable them to breakdown the end products as they form, then a significant improvement in survival may be possible.

The consumption of scallops as a food product has long been associated with seasonal fluctuations in demand (Beeton, 1861; Bamfield, 1991). This seasonality, which reflects product quality, corresponds to well defined seasonal variations in the scallop itself. These natural fluctuations, which relate to feeding and reproductive cycles (Ansell, 1974; Comely, 1974; Taylor & Venn, 1979), are manifest by changes in the storage levels of important energy reserves which consequently affect the taste and texture of the muscle and gonad, the main

edible portions. Hochachka & Mustafa (1972) and de Zwann & Zandee (1972) showed a correlation between high carbohydrate content of intertidal bivalves and their ability to withstand the stress of tidal exposure. Conversely, it seems probable that low reserves will reduce stress tolerance and hence, the effect of environmental factors during emersion will be greater. A link between changes in survival ability and seasonal changes in biochemical composition might also relate to observed consumer trends, since periods of low meat quality would also coincide with the period least likely to result in successful live transport. The relationship between survival and biochemical condition was investigated using the glycogen content of the adductor muscles as a likely indicator of physiological condition, since glycogen is known to be the primary energy substrate utilised by emersed marine bivalves (de Zwann, 1983) and the adductor muscles are the main storage sites.

3.1. Maintenance of experimental animals

Pecten maximus (L.) of shell height 10 ± 1 cm were obtained from the Sea Fish Industry Authority marine farming unit, Ardtoe, Argyll, Scotland. Scallops were transported to the Department of Zoology, Glasgow University in double lined, water-filled polythene bags which had been sealed and inflated with oxygen. Upon arrival, the scallops were maintained in a number of aquaria which were supplied with re-circulating sea water at a temperature of $10 \pm 1^\circ\text{C}$ and salinity of 32‰.

Scallops used for biochemical analysis of muscle tissue were usually prepared within 3 days of arrival and always within 5 days. Scallops used in survival experiments were also utilised within the same time period. All other physiological experiments made use of scallops usually within 2 weeks and never beyond 3 weeks of aquarium storage in order to avoid the effects of nutritive stress (Bayne and Thompson, 1970; Gabbott and Bayne, 1973).

Materials and methods

3.2. Survival chamber apparatus

The method for all survival experiments was kept constant for each trial with only the experimental variable being altered.

Experiments were carried out in constant temperature environments ($\pm 1^\circ\text{C}$), at constant relative humidity (r.h. $95 \pm 2\%$) and in a standardized chamber. Both temperature and humidity were monitored throughout each experiment using a Solomat 455 thermohygrometer. Each chamber consisted of a fibre glass tank (1m x 0.5m x 0.5m) with a detachable lid which could be clipped to the tank rim to form a relatively enclosed environment. Holes were drilled into the lids through which air lines or recording probes could be inserted. When not in use these holes were fitted with rubber bungs. Scallops were placed on racks within the tanks which kept the animals off the tank bottom where a layer of sea water was retained in order to maintain the normal experimental humidity of 95%. Each rack could hold a maximum of 12 scallops, with a maximum of 2 racks per chamber, giving a total of 24 animals in each chamber. The air supply to the tank was first bubbled through a water filled Buchner flask before being fed through diffusing air stones which, when in contact with the water, also helped to maintain humidity by creating an aqueous aerosol. The survival chambers were housed on racks within a constant temperature aquarium (10°C) or in temperature controlled rooms for all other temperatures. Air was supplied to the chambers either from a compressed air supply, or from portable 'Hy-flo' aeration pumps.

3.3. Assessment of survival

The determination of death in organisms such as scallops can be difficult and during these experiments the procedure followed was similar to those employed by Dickie (1958) and Epifanio and Srna (1975).

During emersion in air the shell valves of *Pecten maximus* gape slightly, thus exposing the edge of the mantle lobe, which is rich in sensory receptors such as eyes and tentacles (Stephens, 1978). Stimulation of this area, therefore, results in rapid withdrawal of the mantle edge and shell valve movement. Throughout each experiment scallops were checked every 6 hours, though usually at an 8 hour interval overnight. Several areas on the mantle edge of each scallop were stimulated with a fine glass rod and if no response was noted then death was assumed. After this point was reached, the scallop was removed from the experimental chamber, returned to the re-circulating sea-water system and re-tested at the following 6 hour check. If a negative response was again noted, then death was recorded at the initial time that the scallop was removed from the chamber. If a mantle response was recorded following the re-immersion period then death was recorded at this later time and the animal was removed from the trial. In practice, if no mantle response was recorded initially then a positive response was seldom found following re-immersion. In no case did a scallop recover fully after re-immersion.

3.4. Effect of temperature on survival

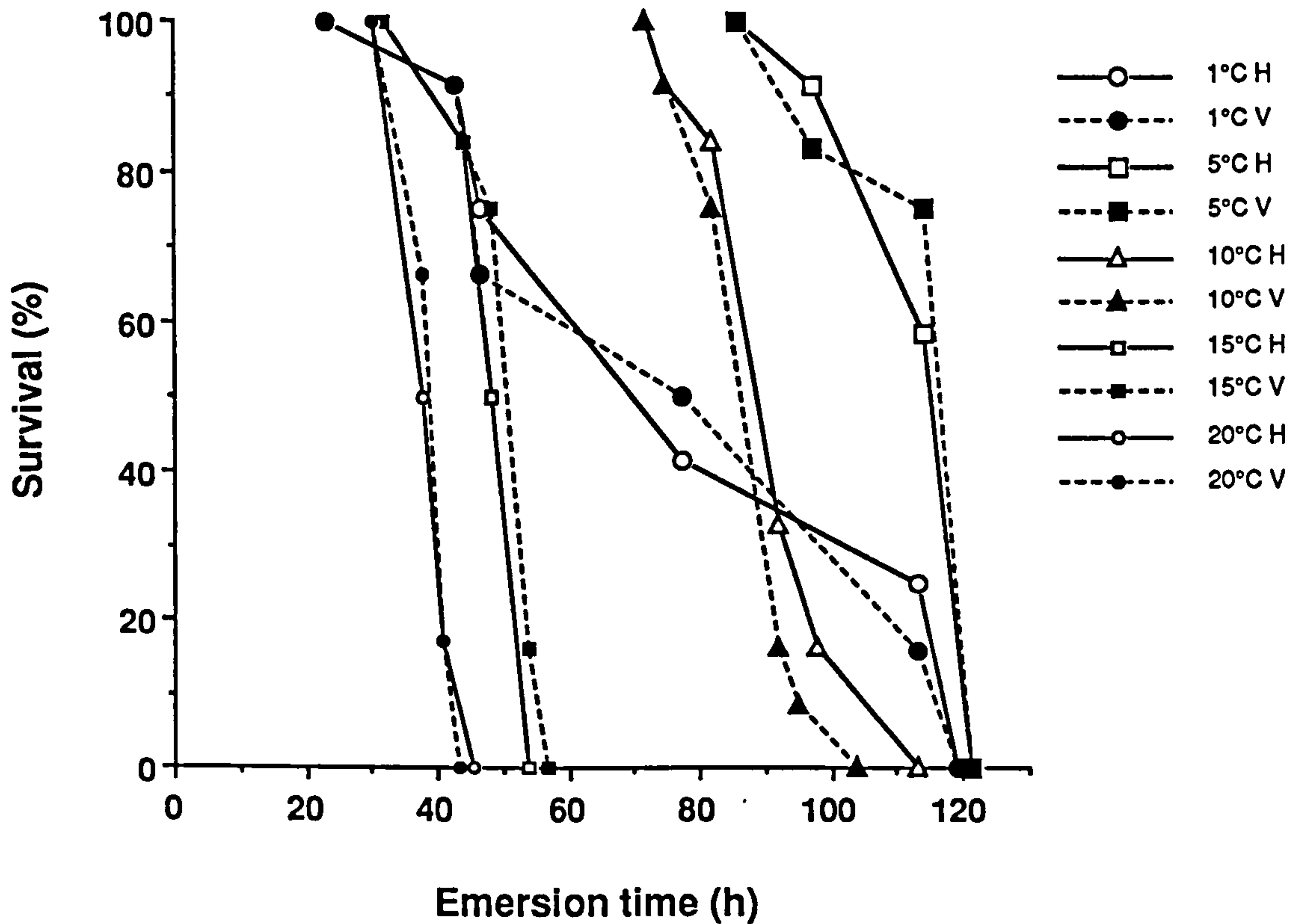
Materials and method

The effect of air temperature on mortality rates and maximum survival time was investigated using constant temperature rooms set at; 20, 15, 10, 5, and 1°C. Relative humidity was kept high at 95 ±2% using the method described in 3.2. Temperature and humidity were monitored throughout the trials using a Solomat 455 thermohygrometer. Twelve scallops, of shell height 10 ±1cm, were emersed at each temperature. All temperature/survival trials were carried out in a random order between March and May in order to minimise any possible seasonal effects on survival ability (see 3.10.).

Results

Survival of scallops during emersion at different environmental temperatures is shown in Fig. 3.1. The maximum survival time of 121 hours was recorded for scallops exposed at an air temperature of 5°C. Survival time decreased as emersion temperature was increased. Maximum

Fig. 3.1 *Pecten maximus*. Survival during emersion at air temperatures of 1, 5, 10, 15 and 20°C. Emersions were carried out in constant temperature environments ($\pm 1^\circ\text{C}$) and at a relative humidity of $95 \pm 2\%$. N=12 at each temperature. Using the Kolmogorov Smirnov two-sample, two-tailed test the differences in survival between temperatures were significant, $P < 0.01$, except between 5 and 1°C where $P = 0.05$. Closed symbols represent scallops stored vertically to allow drainage of mantle cavity water, open symbols represent scallops stored horizontally in order to retain water (see section 3.7), n=12 at each orientation. Differences between vertically and horizontally stored scallops were not significant using the same test, $P > 0.05$.



values of 113h, 57h and 45.5h were recorded for 10°C, 15°C and 20°C respectively. At 1°C maximum survival was found to be 119 hours, although mortalities began much earlier than in the 5°C trial.

The mortality curves were similar in form for all temperatures with the exception of 1°C. All scallops survive for a certain length of time and then die over a relatively short interval thereafter. The typical pattern may be illustrated by observing the time at which the scallops begin to die. At 5°C the first deaths were recorded after 86 h, and all scallops were dead within the following 35 h. The total time for all scallops to die increased to 41 h at 10°C, but fell to 25 h at 15°C and 15 h at 20°C. At 1°C the duration of the mortality period was 96 h, from 23 to 119 hours of emersion.

The differences in survival between each experimental temperature from 20°C to 5°C were statistically significant (Kolmogorov-Smirnov two-sample, two-tailed test (KS2st), $P < 0.01$). The difference between survival at 5°C and 1°C was significant at $P = 0.05$. This statistical test is used to determine whether 2 independent samples have been drawn from the same population by comparing their cumulative frequency distributions, i.e. the proportion of observations which are less than, or equal to, a particular value. If the samples come from the same population, then the cumulative distributions would be expected to be close, in that they will show only random deviation from the common population distribution. If, however, the two cumulative distributions are statistically 'too far apart' at any point then it is assumed that the samples are derived from separate populations. The two-tailed test is sensitive to any kind of difference and does not require that one specific sample is larger than the other in order to obtain a significant difference, for example, testing whether an experimental group is greater than a control group. Further details of this test are described by Siegel & Castellan (1988). The pattern of survival is that at emersion temperatures higher than the optimum, i.e. 5°C, scallops survive for a shorter overall time and die within a shorter period. Emersion temperatures below the optimum also reduces survival significantly.

It is perhaps necessary to explain the rapid decline in survival towards the end of the 5°C experiment. The length of exposure, >110 h, resulted in a rather poor internal appearance of the animals and consequently led to difficulty in assessment of the mantle response. As a result, after 114 hours of emersion some scallops appeared to show no response but were later found to respond following re-immersion. These animals were therefore removed from the experiment earlier than was appropriate. It is probable that maximum survival would be closer to 130-135 h and the mortality period about 50 h.

3.5. Effect of reduced humidity on survival

Materials and method

The effect of humidity on survival was investigated by comparing survival rates at $70 \pm 10\%$ and $95 \pm 2\%$ r.h. Temperature was kept constant at $10 \pm 1^\circ\text{C}$ for both trials. The reduced humidity was obtained by drying the interior of the experimental chamber thoroughly, removing excess water from the shell surface and placing 12 scallops horizontally on dry paper towel. Careful monitoring of humidity with the thermohygrometer and removal of the tissue paper when required (if moistened by water from the inside of the scallop), allowed humidity to be kept to within $\pm 10\%$ of the desired value. Twelve scallops were exposed at $95 \pm 2\%$ r.h. to act as a control group.

Results

Scallops maintained at 95% r.h. began to die between 93 and 100 h, and showed a maximum emersion survival time of 137 h. (Fig. 3.2). Those scallops exposed at the reduced humidity of 70% began to show mortalities before 78 h and had a maximum survival time of 100 h.

The treatments were compared using the KS2st and the results showed that scallops emersed at the lower humidity had significantly reduced survival ($P=0.01$).

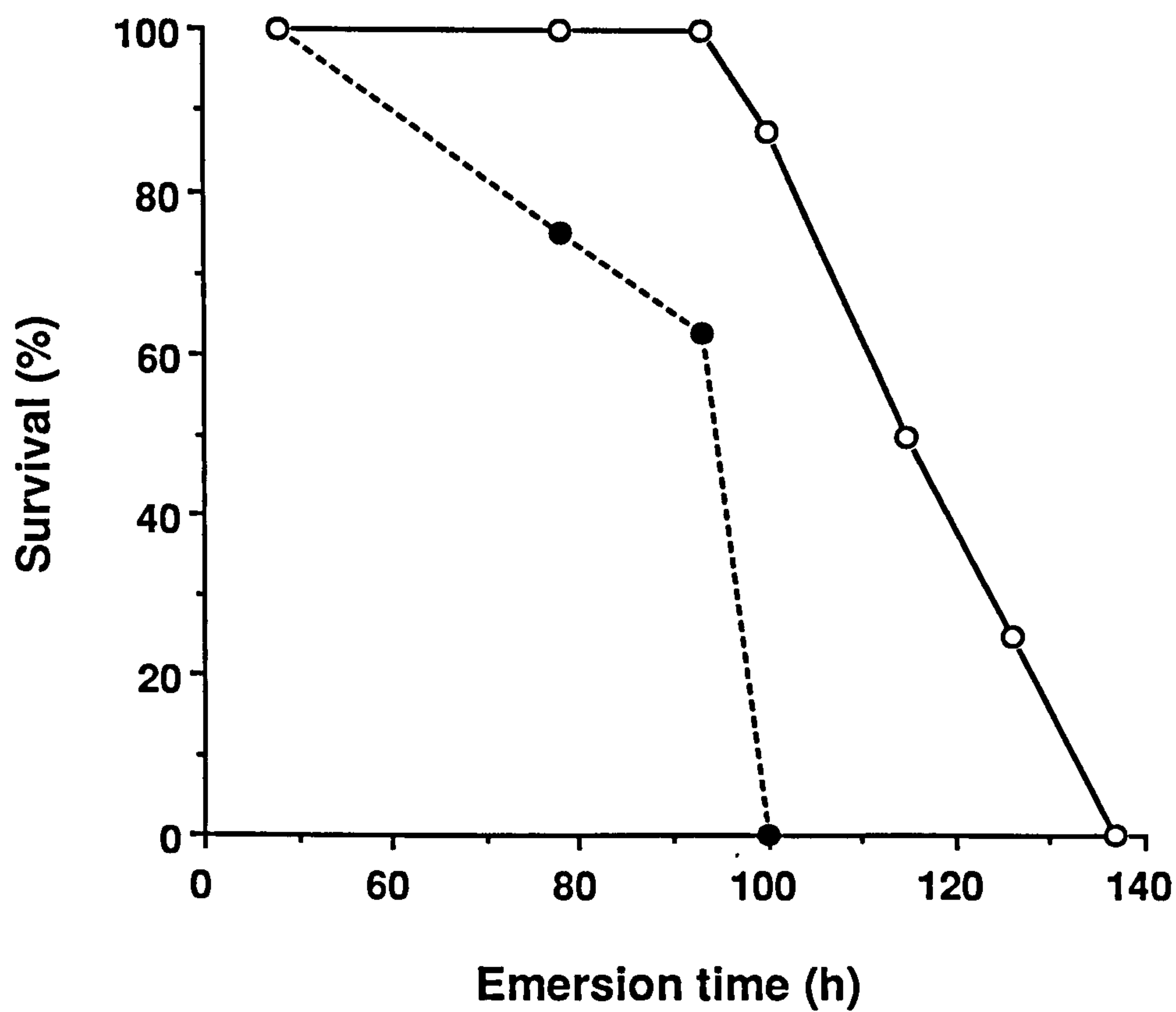
An interesting observation made during this experiment was that the reduction in humidity was sufficient to cause the ligament and hinge mechanism to dry out. Consequently, if the scallops adducted during the trial the movement often caused the joint to break, resulting in dorsal separation of the shell valves.

3.6. Effect of hyperoxia on survival

Materials and method

Eight scallops were placed on a rack within large double lined polythene bags and beneath each rack was a tray containing sea water in order to obtain the humidity conditions experienced in the normal experimental chamber. Humidity was monitored during the experiment and was within $95 \pm 2\%$. An airline, terminating in diffusing airstones, was fed into the neck of the bag which was then constricted around the tubing in two places using cable ties. The external end of

Fig. 3.2 *Pecten maximus*. Survival during emersion at $10 \pm 1^\circ\text{C}$ under conditions of $95 \pm 2\%$ (open symbols) and $70 \pm 10\%$ (closed symbols) relative humidity. $N=12$ for each humidity treatment. Difference in survival is significant using the Kolmogorov Smirnov two-sample, two-tailed test, $P=0.01$.



the airline was attached to an oxygen cylinder and the bag inflated with pure oxygen. Leakage from the bag was minimal, though slight re-inflation was carried out during each 6 hour interval to keep the oxygen concentration as close to 100% as possible. The bag was re-inflated after each assessment period.

A control group of 8 animals were emersed at the same time using the method described in 3.2. Both experiments were carried out in a constant temperature environment of $10 \pm 1^\circ\text{C}$.

Results

Scallops emersed under hyperoxic conditions began to die after 98 h, the last animal dying at 126 h (Fig. 3.3). In comparison, the control group showed slightly better survival during the trial, though maximum survival time was identical at 113 h and the differences observed were not statistically significant.

3.7. Effect of scallop orientation on survival

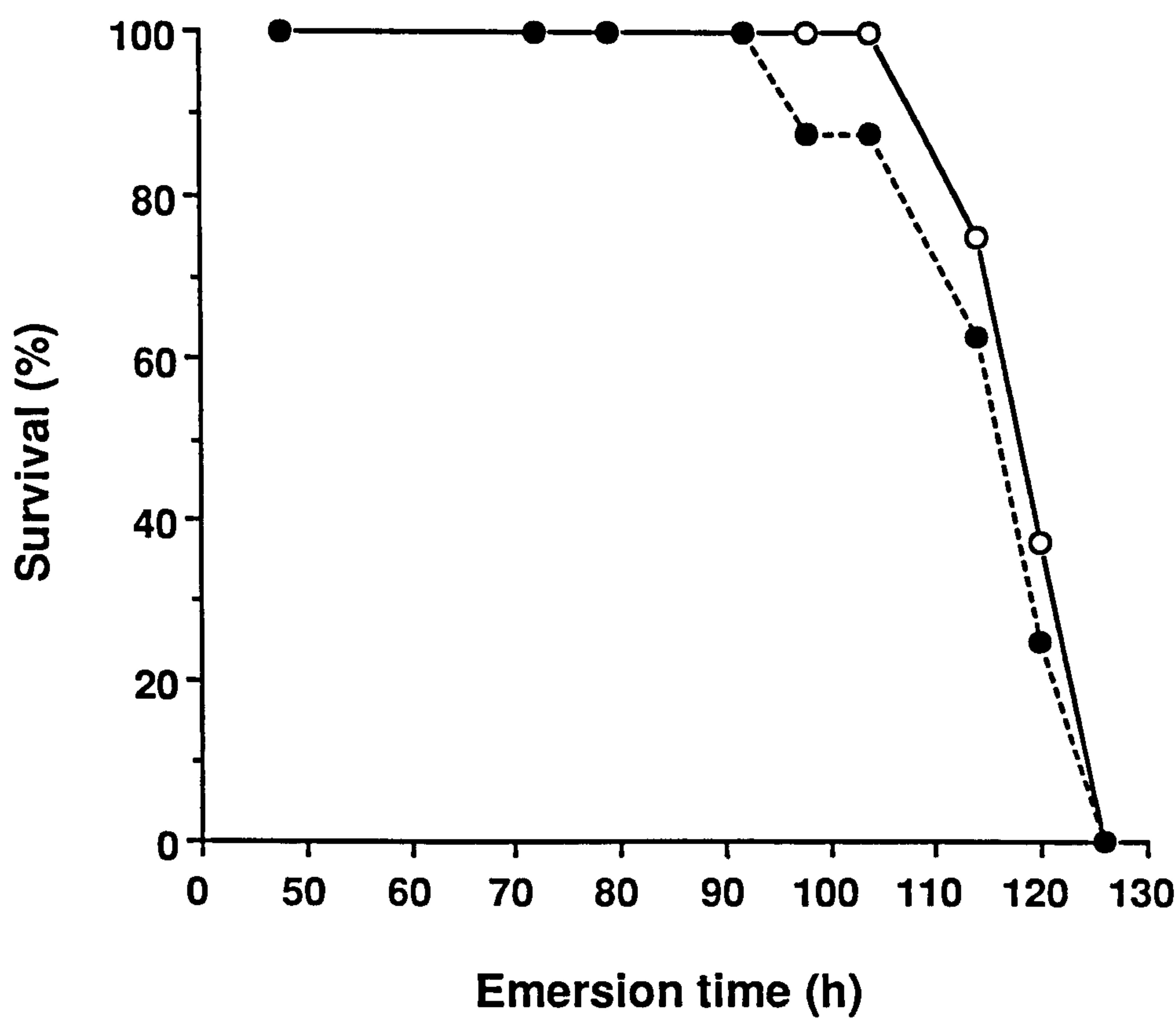
Materials and method

During the temperature survival experiments an additional 12 scallops were emersed at the same time in order to determine the effect of storage orientation on survival. The animals were placed in a vertical position against the sides of the chamber which caused water to drain from the ventral edge of the mantle cavity. Care was taken to balance the scallops on the margin of the right valve, which usually extends beyond the left valve, to allow the animals to adduct without falling over.

Results

At each emersion temperature the difference in survival between scallops stored vertically, i.e. drained of mantle water, and those stored horizontally, i.e. mantle water retained, was tested using the KS2st. No significant differences were found between drained and undrained scallops at any temperature (Fig. 3.1).

Fig. 3.3 *Pecten maximus*. Survival during emersion at $10 \pm 1^\circ\text{C}$ and $95 \pm 2\%$ r.h. under conditions of hyperoxia (ca. 100% oxygen) (closed symbols) and normoxia (open symbols). N=8 for both conditions. Differences in survival were not statistically significant using the Kolmogorov Smirnov two-sample, two-tailed test, $P>0.05$.



3.8. Effect of enforced shell closure on survival

Materials and method

The effect of enforced shell closure on survival was investigated at $10 \pm 1^\circ\text{C}$ and $95 \pm 2\%$ r.h. Twelve scallops were placed horizontally in an experimental chamber with the shell valves held tightly closed with elastic bands. The effect of this was to prevent the normal emersion adduction response and hence retain more water within the mantle cavity.

A control group of 12 scallops was maintained in the same chamber without elastic bands and could adduct freely.

Results

Enforced shell closure had no effect on survival since maximum survival for scallops under both experimental conditions was identical at 133.5 h (Fig. 3.4). The first mortalities were recorded after 79.5 h of emersion in both treatments. There were no statistically significant differences between the two conditions.

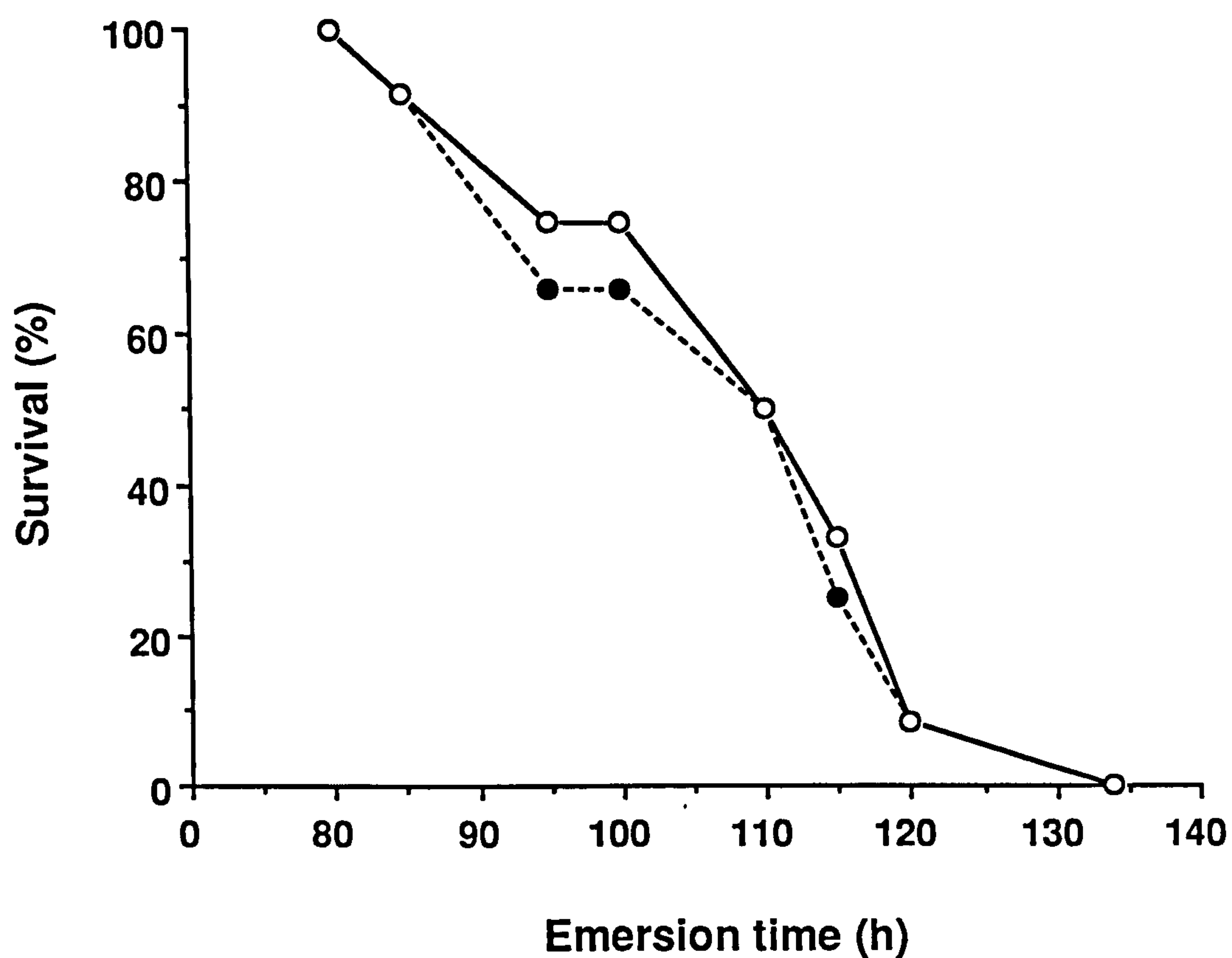
3.9. Misting chamber survival trial

An assessment of a misting transport chamber developed by the Seafish Industry Authority (S.F.I.A.) was carried out at the S.F.I.A. marine farming unit, Ardtoe between 18th and 27th March, 1991.

Materials and method

The misting chamber consisted of a polypropylene box (2m^3) and a removable lid. The underside of the lid was fitted with 6 Lurmark ('full spray jet') sprayheads which were connected to a pump which circulated sea water from a 290 l reservoir. The water was returned to the pump via a drain in the tank base. Water flow rates were variable depending on pump speed and spray head type, in this case the flow rate per head was 60 l hour^{-1} at a pump pressure of 4 bar. Within the chamber a series of plastic trays (50cm x 50cm x 5 cm) (Northwest plastics Ltd.) could be stacked, which in total could hold up to 2000 juvenile scallops (50 per tray) of shell height 5 cm. For the experimental trial, however, a total of 10 larger scallops (shell height $10 \pm 0.5\text{cm}$) were used.

Fig. 3.4 *Pecten maximus*. Survival during emersion at $10 \pm 1^\circ\text{C}$ and $95 \pm 2\%$ r.h. Scallops stored with the shell valves forcibly closed (closed symbols) or with shell valves free to adduct (open symbols). N=12 for each condition. Differences in survival were not statistically significant using the Kolmogorov Smirnov two-sample, two-tailed test, $P > 0.05$.



The scallops were placed in the misting chamber within which air and water temperatures were maintained at $9.5 \pm 1.5^{\circ}\text{C}$. To allow comparison, a control group ($n=10$) was set up in a box chamber as described in 3.2. This chamber was positioned adjacent to the misting chamber to ensure that the temperature regimes were identical. Sample sizes were also identical and observations were made every 6 or 8 hours (see 3.3.).

Results

The first deaths recorded in this experiment occurred in the control box sample at 116 h (Fig. 3.5). Maximum survival in this group was 145 h. Scallop mortalities in the misting chamber were first recorded at 145 h with the final deaths occurring by 166 h. The difference between the 2 treatments was statistically significant (KS2st, $P<0.01$).

3.10. Effect of seasonal factors on survival

Materials and methods

Determination of monthly variation in scallop survival ability was made according to the method described in 3.2. & 3.3. The emersion conditions were $10 \pm 1^{\circ}\text{C}$ and $95 \pm 2\%$ r.h. Scallops were emersed at monthly intervals from November 1991 to August 1992, except for June 1992 when samples were not obtained due to difficulties of specimen supply. Sample sizes ranged from 7 to 10 animals.

During this 10 month period a sub-sample of 3 scallops from each monthly delivery was taken and a muscle glycogen assay carried out. Adductor muscle tissue was removed from the scallop, frozen in liquid nitrogen and stored at -70°C prior to freeze drying and preparation for the assay. Preparation and analysis was carried out according to the method described in 6.4.

Results

The maximum survival time of scallops during emersion varied depending on the time of year and appeared to fall into 3 distinct groups (Fig. 3.6). Survival time tended to decrease from November/December to July and increase thereafter. Actual times are shown in Table 3.1.

Fig. 3.5 *Pecten maximus*. Survival during emersion at $9.5 \pm 1.5^\circ\text{C}$. Lines show comparison between the standard box chamber (see section 3.2)(closed symbols) at $95 \pm 2\%$ r.h. and a misting transportation chamber (open symbols) which delivered a fine spray of sea water. $N=10$ in both chambers. Differences were statistically significant using the Kolmogorov Smirnov two-sample, two-tailed test, $P<0.05$.

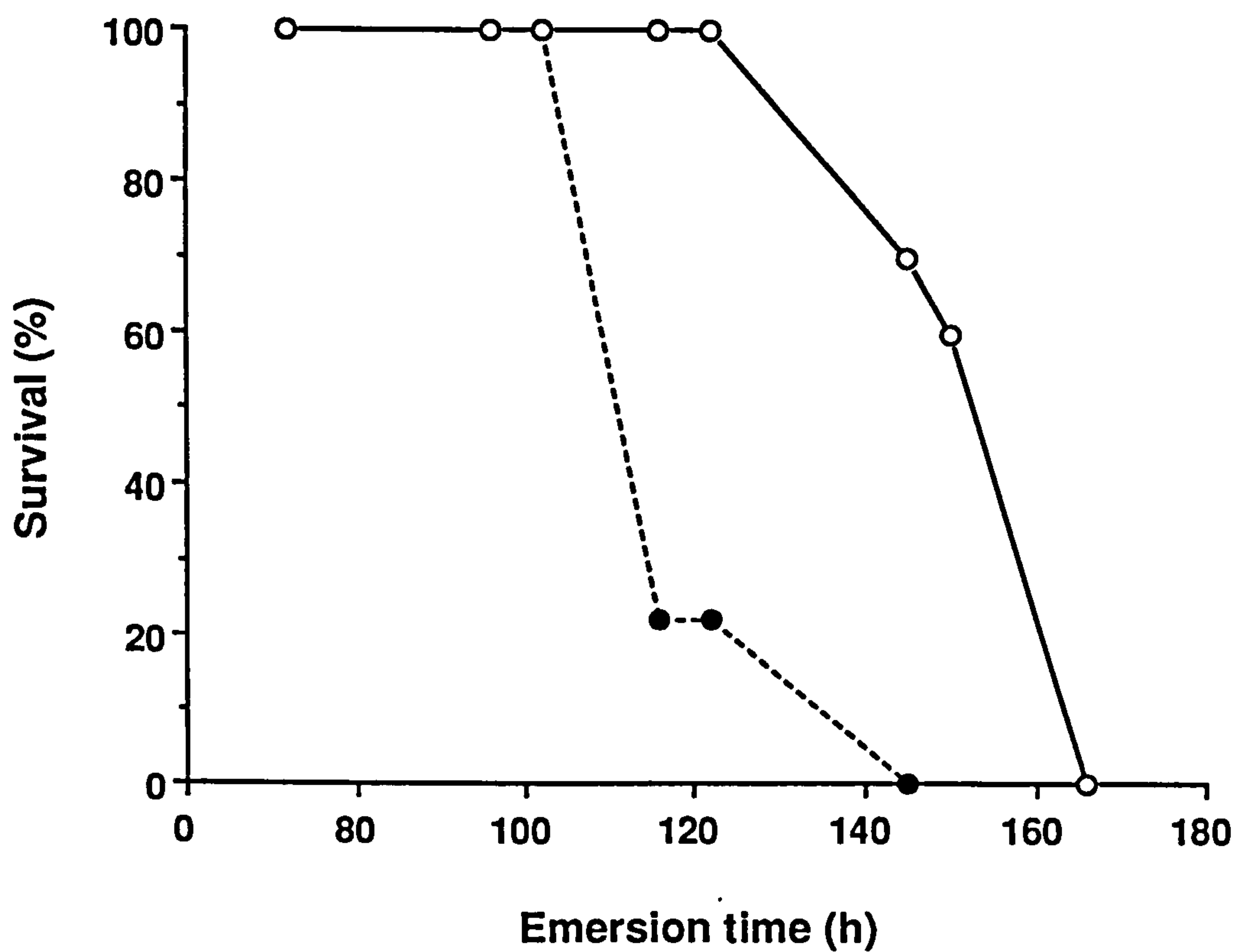


Fig. 3.6 *Pecten maximus*. Survival during emersion at $10 \pm 1^{\circ}\text{C}$ and $95 \pm 2\%$ r.h. Scallops were emersed at monthly intervals between November 1991 and August 1992. Differences in survival were tested using the Kolmogorov Smirnov two-sample, two-tailed test which showed significance at $P < 0.05$ between November/December and February-July and between February-May and July. Comparisons between February and May showed no significant differences. $N=7$ to 10 . June values not obtained.

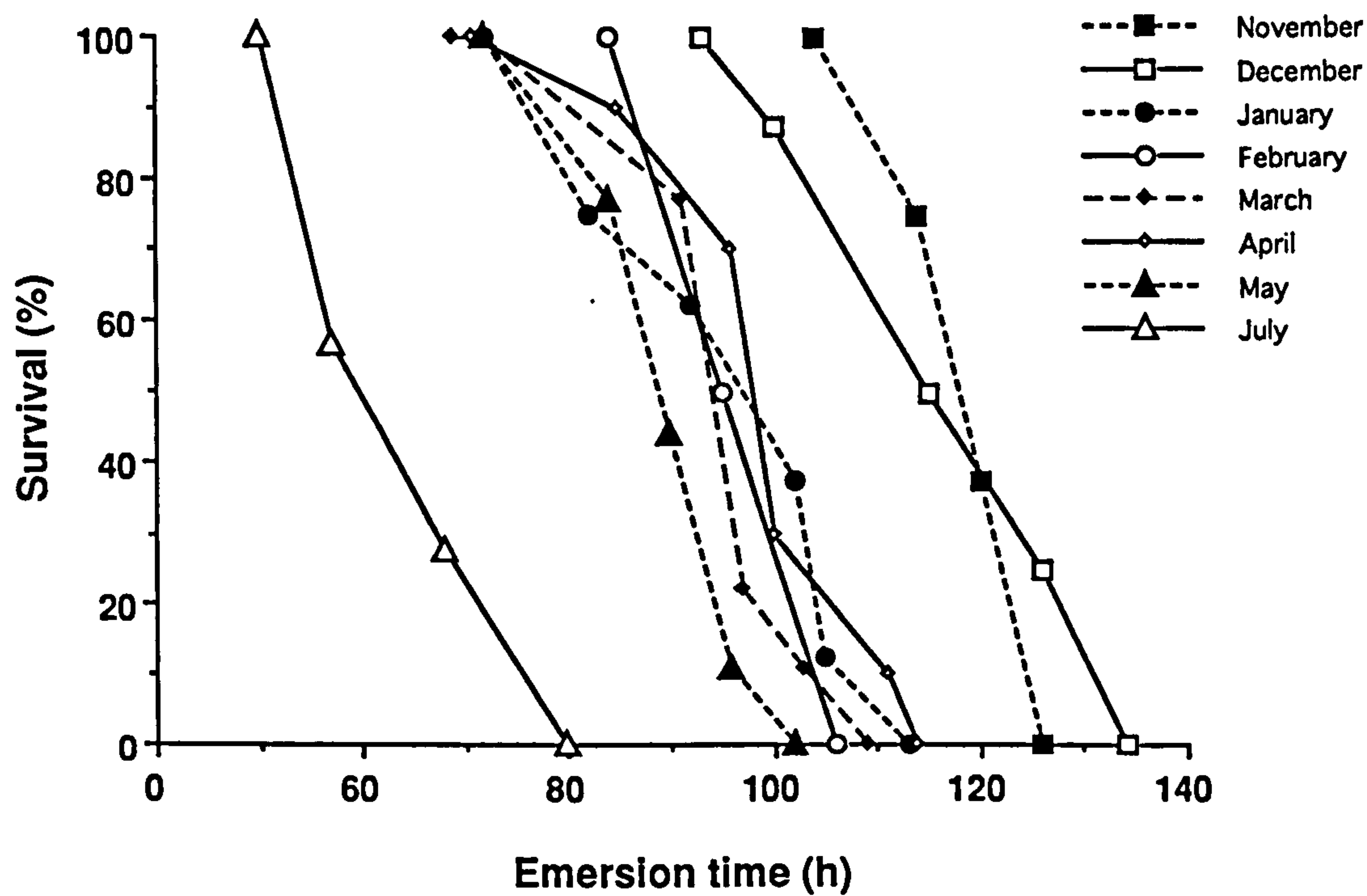


Table 3.1 Survival times obtained from experimental emersion of *Pecten maximus* (n=7 to 10) between November 1991 and August 1992. Scallops were emersed at 10 ±1°C and 95 ±2% r.h. Values shown relate to time of first death, maximum survival time and interval between first and last deaths. * Particular reference is made to August values in the text. June values not obtained.

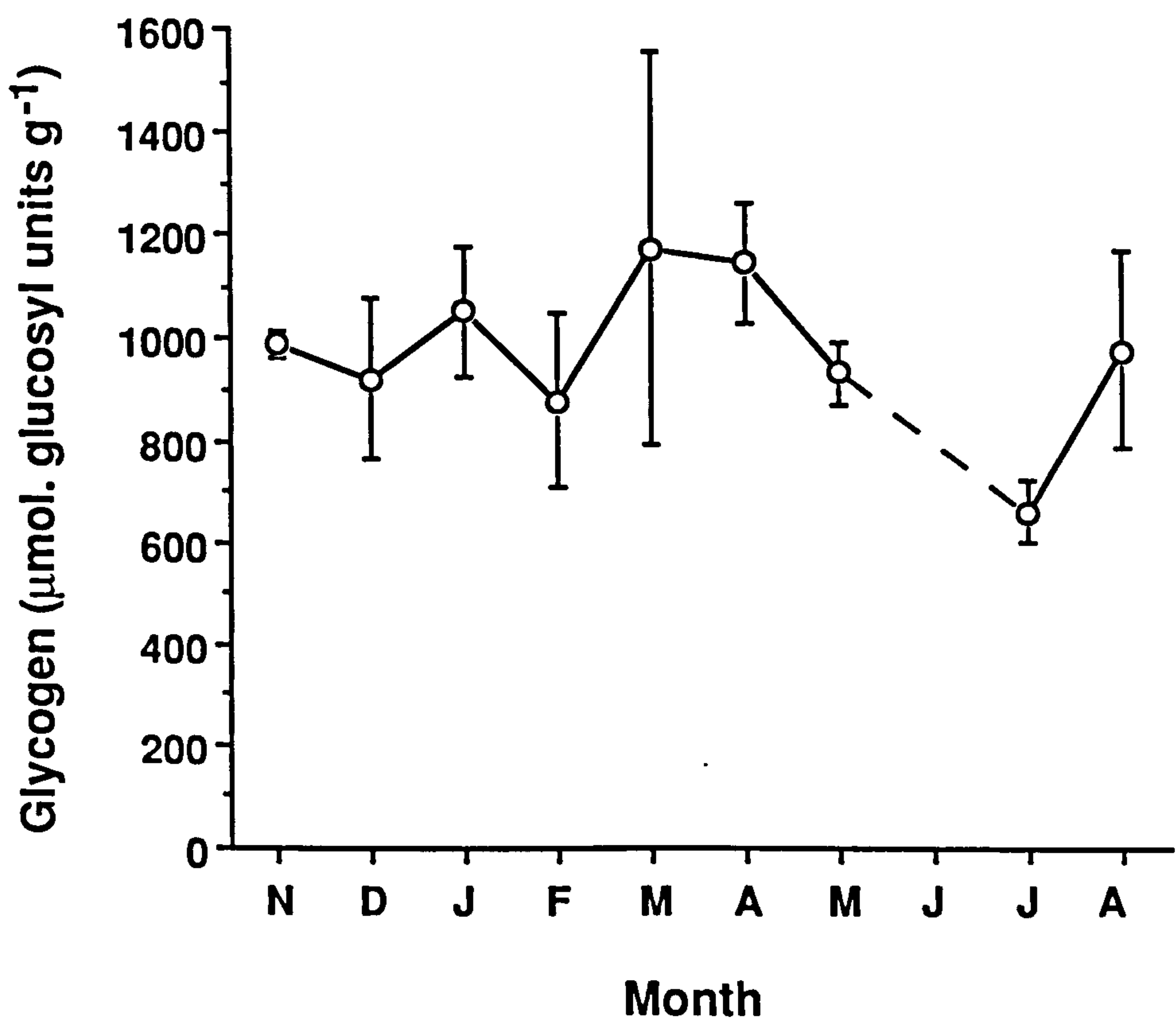
MONTH	FIRST DEATH (h)	MAXIMUM SURVIVAL (h)	MORTALITY PERIOD (h)
November	104	126	22
December	93	134	41
January	72	113	41
February	84	106	22
March	72	109	37
April	71	114	43
May	72	102	30
July	50	80	30
August*	56	96	40

The time of first death also showed a decline over the same period, from 104 h in November to 50 h in July. Mortality periods, i.e. the interval between the first and last death, were broadly similar for each month, except November and February. Statistical analysis of the data using the Kolmogorov-Smirnov two-sample, two-tailed test showed no significant difference between November and December survival experiments. The November data were significantly different from January data ($P<0.05$), although the December data were not. By February, however, survival time was significantly lower compared with both November ($P<0.01$) and December ($P<0.05$). Between January and May survival was similar, but the large decrease in survival observed in July was statistically different from these 5 preceding months ($P<0.05$, except February where $P<0.01$).

Values for August were recorded, although unfortunately, in a rather limited form. These data are shown in Table 3.1. but have been omitted from Fig. 3.6. Missing values prevented statistical comparison with other months but it was apparent that survival had improved from 80 h in July to 96 h in August. The time recorded for the first deaths also increased from 50 to 56 h between July and August.

Muscle tissue glycogen concentrations showed no statistically significant differences between November ($987.8 \pm 24.7 \mu\text{mol. (glucosyl units) g}^{-1}\text{dry weight}$) and May ($931.0 \pm 60.6 \mu\text{mol. g}^{-1}$) (ANOVA, $P>0.05$) (Fig. 3.7). July samples, however, showed a decrease in total

Fig. 3.7 *Pecten maximus*. Variation in adductor muscle glycogen concentrations between November 1991 and August 1992. Data shown are means (\pm S.D.) of total muscle concentrations (phasic and tonic adductors) in $\mu\text{mol. glucosyl units g}^{-1}$ dry tissue weight. N=3 for each month. Differences in glycogen concentration were not significant between November & April, but were between April & May, $P<0.05$ (ANOVA), April & July, $P<0.05$ and November & July, $P<0.01$. August values were not significantly different from November-May values. June values not obtained.



muscle glycogen to $658.3 \pm 62.1 \mu\text{mol. g}^{-1}$ which was significantly different from the November value (ANOVA, $P < 0.01$). Both May and July values also differed significantly from the April value (ANOVA, $P < 0.05$). By August, muscle glycogen levels had increased again ($973.0 \pm 191 \mu\text{mol. g}^{-1}$) in comparison to July, although the change was not quite significant (ANOVA, $P = 0.053$). August values were not significantly different from the November-May values. Over the sample period, therefore, muscle glycogen did not appear to vary except for a decrease between April and July, which was restored again by August.

Discussion

Temperature has an important influence on the survival of *Pecten maximus* during emersion. An increase of 15°C, from 5 to 20°C, caused a reduction in maximum survival of 62.4 or 64%, dependant on the storage orientation of the scallops. Lowering emersion temperature to 1°C did not improve survival, and appeared to cause earlier mortalities, thus overall survival was poorer. It is concluded, therefore, that the optimum emersion temperature, in terms of survival, lies between 2 and 5°C.

Prochazka & Griffiths (1991) determined a similar optimum transit temperature of 4°C for the mussels, *Mytilus galloprovincialis* and *Choromytilus meridionalis*, although over a slightly more limited range of temperatures than the present study. Seaman (1991) also reported the effect of emersion temperature on survival of the oyster, *Crassostrea gigas*. Although no optimum storage temperature was determined for this species, an interesting difference in response to exposure temperature was identified between different size classes. Juvenile oysters showed poorer survival at 0°C but better survival at 7°C in comparison with adults. This observation confirms that, while temperature itself is important in survival during emersion, its effects are influenced by a number of other factors such as body size and metabolic rate.

As temperature rises within the tolerance range of the animal, the metabolic rate tends to increase due to increased reaction rates (Kinne, 1970). This relationship holds both in water and in air and has been demonstrated in many molluscan species (Lubet & Chappuis, 1967; Hilbish, 1987) and (Trueman & Lowe, 1971; Boyden, 1972a; Griffiths, 1981). Some molluscs have been thought to show thermal independence of metabolism (Newell, 1979; Bayne & Newell, 1983), although this was found to apply only to the basal metabolic rate of a quiescent animal. Whether an emersed, adducting scallop should be considered to have a quiescent or an active metabolism is a question which will be addressed in later work, but for the purposes of this discussion it is worth noting that Newell (1966), found no evidence of metabolic thermal independence in sub-littoral species suggesting that this phenomenon may be linked to other littoral adaptations. In general, therefore, (and certainly in an active, sub-littoral species) metabolism increases with increasing environmental temperature until the upper levels of thermal tolerance, or upper lethal temperature, is reached. Metabolic parameters then decline rapidly as death occurs.

What then determines upper lethal temperature in a species or individual and how does this temperature result in death? Inter-specific thermal tolerance depends mainly on adaptive and behavioural responses, particularly during aerial exposure. Intra-specific differences in bivalves vary, depending on developmental stage, age, acclimation or normal environmental temperature,

physiological condition and season (Kinne, 1970). Paul (1980), reported an upper lethal water temperature between 19 and 24°C for *Aequipecten opercularis* and Dickie (1958) found a similar range of 20-24°C for *Placopecten magellanicus*. Thermal tolerance has also been investigated in other bivalves such as *Lima scabra* (Read, 1967), *Modiolus modiolus* and *Brachidontes demissus* (Read & Cumming, 1967) and *Mytilus edulis* (Wells & Gray, 1960). Read & Cumming (1967) estimated limiting sea-water temperatures of 23°C and 40°C for *M. modiolus* and *B. demissus* respectively and, additionally, found that *M. edulis* had an intermediate limit of 27°C. Given that *B. demissus* inhabits the high intertidal zone, it is tempting to suggest that high thermal tolerance is also an adaptation to emersion. This hypothesis is supported by the relatively low thermal tolerance found in extreme low shore or sub-littoral species, such as *M. modiolus*, whereas the mid-shore mussel, *Mytilus edulis*, exhibits an intermediate tolerance.

Thermal tolerance may also vary within a species depending on the time of year and the effect of other factors which alter the influence of temperature. Dickie (1958) reported that the scallop *Placopecten magellanicus* was less susceptible to high temperature in winter and spring than in summer and autumn. This result is perhaps surprising, since the same study also showed that scallops maintained for a long period at higher temperatures, i.e. acclimated to high temperature conditions, had correspondingly higher lethal temperatures. It might, therefore, have been expected that scallops acclimated to the relatively higher water temperatures of summer and autumn would have shown a greater tolerance to raised experimental exposures. This increase in thermal tolerance caused by acclimation has also been shown for various fish species (Brett, 1944; Hart, 1952), but the results obtained for the scallop suggested that other factors, such as physiological condition, may be equally important in the determination of temperature effects. This point will be discussed in more detail later in this section.

Other variations in thermal tolerance shown within and between species are related to differing abilities to withstand the effects of temperature on metabolism. As mentioned earlier, higher temperature may result in increased metabolism. However, higher temperature water has a lower concentration of dissolved oxygen to supply this requirement (Weiss, 1970) and, in bivalves, higher air temperatures cause an increased oxygen demand from a respiratory system primarily adapted for an aquatic environment. Consequently, in water, bivalves must increase the water flow rate over the gills to deliver sufficient oxygen to the tissues. The beating rates of cilia, which generate the respiratory currents, are maximal in *Modiolus modiolus* and *Mytilus edulis* at 20° and 26°C respectively (Schlieper *et al.*, 1958) and decrease at higher temperatures. Comparison with estimates of thermal tolerance for these species (23° and 27°C) suggests that, at these temperatures, oxygen uptake may be impaired and process integration, such as ciliary beating, is affected (Kinne, 1970).

Aerial exposure of bivalves at high temperatures compound these respiratory problems since, in addition to the higher energy demand, the gas exchange surfaces, such as the mantle surface or gills, are also permeable to water and the risk of desiccation is added to the difficulties of aerial respiration. Dealing firstly with respiration in air, Hochachka & Somero (1973) defined 3 potential strategies.

- 1) Compensatory
- 2) Exploitative
- 3) Avoidance

The first strategy depends primarily on the short term production of energy via anaerobic pathways during temporary periods of low oxygen availability, e.g. tidal emersions. Such a strategy is shown by *Mytilus edulis* (Widdows *et al.*, 1979) and *M. californianus* (Bayne *et al.*, 1976). Both mussels typically close their shell valves, reduce their oxygen consumption and, over the tidal cycle, build up anaerobic metabolites which are subsequently broken down during repayment of the oxygen debt following re-immersion.

An exploitative strategy, frequently found in benthic and parasitic invertebrates, may also be encountered in intertidal bivalves. Animals employing such a strategy are able to utilise anaerobic pathways for extended periods by linking glycolytic phosphorylation to other substrate-level phosphorylations resulting in an increased yield of ATP under anaerobic conditions. This form of energy production may account for the impressive ability of the cultured oyster, *Crassostrea virginica*, to survive aerial storage for up to 6 months (Medcof, 1959).

The final strategy encountered in emersed bivalves also results in an ability to withstand prolonged exposures, although the pathways are exclusively aerobic. Bivalves inhabiting the high-littoral zone, such as *Polymesoda caroliniana*, are capable of obtaining sufficient oxygen to maintain aquatic respiratory rates in air (Deaton, 1991). They incur no oxygen debt and therefore show no build up of toxic anaerobic metabolites. Such adaptation allows colonization of areas which may be immersed for as little as 3.2% of the time.

In general, therefore, bivalves adapted to a littoral environment maintain aerobic metabolism during emersion, whereas low littoral and sub-littoral species close their shell valves and resort to anaerobic pathways (Widdows *et al.*, 1979).

The situation in *Pecten maximus* is rather unusual since, as a sub-littoral bivalve, it might be expected to follow the latter strategy. Such is the case with 2 other truly sub-littoral species,

Cerastoderma glaucum (Boyden, 1972b) and *Modiolus squamosus* (Nicchitta & Ellington, 1983). However, *Pecten maximus* exhibits repeated valve adductions during emersion, a feature associated with mantle cavity ventilation and air breathing (McMahon, 1988; Byrne *et al.*, 1990), and it does not seal its shell valves for any extended period. Indeed, observations suggest that valve gaping actually occurs, although it is unclear whether this is an active process or a result of muscle fatigue following adductions. This pattern is similar to that shown by bivalves, such as *Cerastoderma edule*, which are considered to be well adapted for the intertidal zone. In this species, valve gaping and adduction were associated with air breathing which enabled it to survive for up to 289 hours (LD₅₀'s at 10°C), compared with only 220.5 hours for *C. glaucum* which remained closed and used predominantly anaerobic metabolism (Boyden, 1972b). Valve gaping and adductions which ventilate the mantle cavity appear to be beneficial to emersed bivalves, yet *Pecten maximus* survived under equivalent conditions for only 90 hours.

The question is posed that, if emersion behaviour in the scallop is at least superficially similar to that shown by predominantly aerobic, air-breathing bivalves, what physiological differences might explain the dramatic variation in survival ability? A more complete answer to this question requires quantitative measurement of both aquatic and aerial respiratory rates which are examined in the following chapter.

Hyperoxic conditions were found not to improve aerial survival of *Pecten maximus*. Possible explanations for this result are that either the scallop is not using oxygen, and hence aerobic pathways, or that it is incapable or inefficient in the uptake of oxygen across respiratory surfaces. The first hypothesis is perhaps less probable, since valve gaping in air is not in keeping with the usual anaerobic strategy exhibited by emersed bivalves. This explanation, however, cannot be entirely ruled out until actual respiratory rates have been measured.

The second proposal, that of insufficient oxygen delivery, fits the observed behavioural reactions to emersion and may have an explanation in the morphology of the scallop. Solis & Heslinga (1989) found that exposure to 100% oxygen significantly improved survival of juvenile *Tridacna derasa*, although only when the emersion period was more than 24 hours. Emersions up to 48 hours duration gave a survival improvement of almost 79% and they concluded that the clams were relying primarily on aerobic respiration with oxygen being absorbed across the mantle. Juvenile specimens, however, have a proportionately larger gas-exchange surface available and it was noted that hyperoxia was not as beneficial to larger animals. Thus, the main surface for gas exchange in exposed bivalves is the mantle, and in particular the mantle fringes (Boyden, 1972b). The mantle lobes of *Pecten maximus* are particularly thin and, with their fine vascular system, Dakin (1909) considered them to be the most important organs of respiration for the immersed scallop. Although, how efficient the

system would be if it had to supply tissues during emersion remains unclear, especially since both Kuenzler (1961) and Lent (1968) suggested that available surface area for gaseous exchange was a limiting factor for aerial respiration in *Modiolus demissus*, a species supposedly well adapted for emersion.

Bivalves in general are not well adapted structurally for gas exchange in air (Lent, 1968) and the main reason for this, particularly within the Lamellibranchia, is that the gills are more specialised for feeding rather than respiration (Ghiretti, 1966). Although suitable for gas exchange, on account of their large surface area and thin, convoluted epithelial layer (Beninger & Le Pennec, 1991), the secondary nature of the gills respiratory function may be illustrated by the findings of Booth & Magnum (1978) who showed that, by ligation of the anterior aorta of *Modiolus demissus*, aquatic oxygen consumption decreased by only 15%. If this is also the case in scallops then the rather thin mantle lobes, supplied with very fine blood vessels (Beninger & Le Pennec, 1989) may be insufficient to meet the respiratory requirements of the emersed scallop. Alternatively, if greater reliance is placed on the gills, then lack of support for the relatively large surfaces, and consequent clumping of the filaments must also limit their efficiency as respiratory organs.

Consideration of the results of the storage orientation and enforced shell closure experiments may also help determine the direction of later work. Traditional packing methods do not appear to confer any survival advantage on the scallops, although retention of mantle cavity water may help to avoid desiccation in less than optimum humidity conditions. It does not appear to reduce mortality due to any of the potential respiratory explanations proposed in the introduction. This may support the hypothesis that the gills are not the primary organs of aerial gas exchange since prevention of filament clumping, by provision of mantle cavity water, might then be expected to confer some advantage. Alternatively, any benefit of mantle water may be offset by the likely colonization of the fluid by bacteria, which multiply more efficiently in the aqueous environment and are likely to remove at least some of the available oxygen before the gills. The effects of microbial activity on survival during emersion will be dealt with in chapter 5.

Similarly, enforced shell closure resulted in no survival benefit or penalty. It was thought that this treatment would reduce air penetration of the mantle cavity and hence reduce survival by limiting aerobic respiration. The findings indicate that, in common with the hyperoxia experiment, actual oxygen availability might not be limiting, but rather the mechanism to absorb and distribute the gas. Consequently, it is likely that other forms of metabolism are being utilised. The absence of improved survival under different regimes of oxygen tension, storage orientation and enforced shell closure have, therefore, a number of compelling explanations

which require more detailed investigation of the nature of respiration and metabolism in air. This work will be detailed in chapters 4 and 6.

Desiccation is a major factor influencing the survival of scallops during emersion. Exposure to reduced humidity (approximately 70% compared with 95% r.h.) caused a decrease in maximum survival of 27%. Water loss is considered to be important in littoral zonation and an ability to resist or control desiccation allows an organism to survive longer exposures and hence colonise higher levels of the shore (Kensler, 1967). Since high-shore bivalve species open their shell valves in order to maintain respiratory levels, water loss is unavoidable, and species such as *Modiolus demissus* can survive water loss of up to 38% of body weight (Lent, 1968). However, valve gaping is controlled to gain maximum benefit from gas diffusion and minimum effect from desiccation, thus, survival during emersion is maximised. Less well adapted species such as *Mytilus edulis* avoid the conflicting problems of water loss and gas exchange by sealing their shell valves and resorting to anaerobic metabolism for the duration of the emersion period and desiccation is negligible (Coleman & Trueman, 1971; Coleman, 1973). In contrast, the same studies investigated *Modiolus modiolus*, an extreme low shore bivalve, and found that, in common with *Modiolus demissus*, this species also gapes during emersion. However, in this case significant and rapid water loss was observed, largely via drainage from the mantle cavity and evaporation due to uncontrolled exposure of moist permeable tissues. This behaviour, and subsequent desiccation was considered significant in terms of survival, particularly when the effects were enhanced by wind action.

Lopez & Heslinga (1985) also found that desiccation was a factor affecting the survival of juvenile *Tridacna derasa* during emersed transportation. In this case removal of the byssal apparatus unplugged the byssal notch or orifice which resulted in drainage of the mantle cavity water and a reduction in survival of over 50%. Since water loss was an important factor influencing survival they recommended retention of the mantle water and transit in humid conditions. It is interesting to compare these results with those determined in this study which suggested that drainage of the mantle cavity was not significant for survival provided that very high humidity was maintained.

Desiccation may cause death in a number of ways. Loss of water from the internal surfaces used for aerial respiration will severely reduce the efficiency of gas dissolution and diffusion, leading to a reduction in overall respiratory efficiency. Equally, removal of CO₂ by the same diffusion process (de Zwann & Wijsman, 1976) would be retarded with the consequent reduction in pH and subsequent effects on biochemical function. Water loss from body fluids may also increase the viscosity of extra and intra-cellular fluids (Kinne, 1963) and thus impair respiratory function by causing inefficient circulation and increased demands on the heart.

Coleman & Trueman (1971) also identified cardiac efficiency as vulnerable to desiccation, primarily through inadequate oxygen delivery and osmotic imbalance affecting cellular function.

Pecten maximus shows no obvious means of controlling water loss and appears to have a typical 'sub-littoral resistance' to the effects of desiccation. Indeed, whilst lack of adaptation to allow sustainable aerial respiration, e.g. failure to control valve gape, might have been expected, the behaviour of valve adduction which increased water loss shows how poorly adapted this species is for natural emersions. Maintenance of high humidity is therefore of importance during transportation, particularly over long distances when a relatively small reduction in this parameter can significantly decrease survival.

Exposing emersed scallops to a fine sea-water mist was found to improve maximum survival by 12.6%, although this figure belies the effect of this treatment during the whole experiment which generally showed a greater difference (Fig. 3.5.). Irrigation of other bivalve species during aerial exposure has also been shown to improve survival (Seaman, 1991; Prochazka & Griffiths, 1991). In the latter study, mortality of *Choromytilus meridionalis* was reduced by reimmersion between post-harvest handling and exposure, while in the former study, the oyster, *Crassostrea gigas*, was found to have switched from anaerobic to aerobic metabolism as a result of irrigation during a 20 week experimental emersion. The control group, which remained anaerobic throughout, experienced total mortality.

Both studies appear to suggest that, while anaerobiosis may be the usual short-term emersion solution adopted by intertidal bivalves, since it is an energy conserving strategy, problems may be encountered if the exposure is extended or the animals are stressed prior to emersion. If a short term strategy is extended then the build up of anaerobic metabolites may be greater than the natural tolerance of the animal, thus resulting in death (Hummel *et al.*, 1989). If, however, the bivalve is constantly irrigated then it may return to aerobic pathways, breaking down the accumulated metabolites and surviving a longer than expected period of emersion. Similarly, animals stressed by harvesting or handling immediately prior to emersion will have a raised metabolic rate as a consequence of stress. If anaerobic pathways are the only available options, then a more rapid build up of these toxic metabolites, due to the increased metabolism, will result in a more rapid demise. Rewatering between handling and transit allows a return to basal activity, removal of stress induced metabolites and consequently improved survival when the animals are eventually emersed for transit.

Enforced shell closure, in addition to its potential respiratory significance, was also an attempt to limit valve adductions in order to reduce metabolite build up in the active muscle tissue. Gäde (1983) recognised this 'functional anaerobiosis' as being much more significant

than quiescent or 'environmental anaerobiosis' in terms of energetic cost and hence end-product accumulation. However, since no significant survival benefit was derived from this treatment it is possible that muscular activity was still occurring, as 'isometric-like contractions', and that metabolism was not greatly reduced and so little energy was actually saved.

It seems possible, therefore, that accumulation of metabolites, mainly organic acids from anaerobic pathways (de Zwann & Wijsman, 1976), CO₂ from aerobic pathways or ammonia from nitrogen metabolism, could be a significant factor in mortality of emersed bivalves. The effects may be reduced by irrigation, allowing breakdown or flushing of these products. The nature and extent of metabolite build up is worthy of further study and will be dealt with in subsequent chapters.

Survival of scallops during emersion also has a seasonal component. Survival is significantly better in November/December than the following months and by July maximum survival had decreased by 40%. After July there appeared to be a slight recovery in survival ability.

The cause of seasonal changes in survival ability is probably related to variation in the ability of the animal to adjust physiologically to the environmental conditions which affect survival during emersion. Variation in this ability is likely to be related to a seasonal fluctuation in 'condition index', a parameter defined by Hummel *et al.* (1989) as a dry weight per volume ratio. In bivalve molluscs, particularly pectinids, the most noticeable fluctuations occur in the concentration and distribution of energy reserves related to winter metabolism and gametogenesis (Ansell, 1974; Taylor & Venn, 1979). Of particular importance in this regard are the adductor muscles which act as a storage site for carbohydrate and, to a lesser extent, protein. Concentrations of these substrates normally reach a maximum in late autumn/early winter and subsequently decline towards spring, largely as a result of sustaining the animal during winter and mobilising the energy reserves during gonad development (Comely, 1974). These fluctuations, which may result in a 20 fold difference in substrate concentration (Ansell, 1978), can be observed as changes in the average muscle weight (Duncan, 1989), or, when reserves are at their lowest, as a rather watery texture to the muscle.

This reported pattern of fluctuation in biochemical stores relates quite well to the observed monthly survival rates, with optimum survival being found in November/December when reserves should be high after feeding throughout summer and autumn. As the reserves dwindle during winter and early spring, survival showed a general deterioration although this decline continued for longer than might be expected. The apparent decline in both survival and muscle glycogen up to July may be explained by the spawning and/or culture conditions of the experimental scallops.

The seasonal fluctuations described in the above studies relate to the Clyde Sea area where spawning occurs in late spring/early summer, with another possible release in August or September (Comely, 1974). Further south, around the Isle of Man, Mason (1958) reported spawning in spring, a smaller release in summer and the main spawning in autumn. Hence, variation in spawning is also likely to be reflected in the related biochemical reserves. With this in mind, Ardtoe scallops in 1992 were thought to have had their main spawning in late June or early July (J.T. MacMillan, pers. comm.) which may account for the extended decline in glycogen, and hence survival ability, since reserves were still being used for gamete production and maturation. Evidence to support this view comes from the recent work of Eertman *et al.* (1993), who reported that survival of *Mytilus edulis* during emersion was lowest during spawning, a period which also coincided with the lowest glycogen content.

It is surprising, however, given that these fluctuations in biochemical reserves are so well documented, that no significant changes in muscle glycogen were recorded over the period from November to April. Possible explanations are that the relatively small sample size and large individual variations acted to mask the changes during this period. Alternatively, while every effort was made to transport scallops as quickly and naturally as possible from the supply site, disturbance adductions during transit may actually have affected glycogen levels to lesser or greater degrees, again obscuring trends. In addition, the effect of suspended culture on the feeding cycles and subsequent energy storage are unknown and may be significant in producing a different pattern from that observed in Clyde populations. That said, whether the more northerly Ardtoe scallops genuinely have a different pattern of glycogen storage, or whether sample size or transit conditions have obscured subtle trends, it can be assumed that measured substrate levels are accurate in relative terms. In this case the correlation between muscle glycogen concentration and emersion survival does appear to hold.

Consequently, by tradition, scallops were 'out of season' from May until September when biochemical reserves were lowest, the main edible portion (adductor muscle) was least appealing and, as this study has shown, the animals were less able to survive aerial emersion. Such conventions concerning food are, perhaps, less relevant today on mainland Britain since transit time and storage conditions are much improved, but they are clearly important if long haul, live transport to the continent is to be viable.

Chapter 4 Respiration and metabolism during emersion

Introduction

Some of the important physiological problems which directly affect survival of emersed scallops are caused by increased temperature, perhaps affecting metabolism, respiratory dysfunction and the accumulation of toxic metabolites. Increased temperature has been shown to cause an increase in the metabolic rate of many bivalve species when immersed, as implied from parameters such as oxygen consumption and heart rate (Lubet & Chappuis, 1967; Lowe & Trueman, 1972; Widdows, 1973; Griffiths, 1981; Hillbish, 1987). During emersion, however, the relationship between temperature and metabolism may differ depending on activity level (Newell, 1973; Boyden, 1972a; Coleman, 1973), nutritional status (Davies, 1967; Widdows, 1973; Coleman, 1976) and the degree of physiological adaptation to exposure (Helm & Trueman, 1967; Trueman & Lowe, 1971; Widdows *et al.*, 1979; Deaton, 1991).

For example, some high littoral species, which may be emersed for most of their lives, are capable of meeting the oxygen requirements for aerobic metabolism over a wide range of temperatures, largely due to the efficiency of their gas uptake system (Deaton, 1991). Another adaptation is the capacity of organs, such as the heart, to continue functioning even when subjected to rapid changes in environmental conditions (Trueman & Lowe, 1971). Thus, physiological adaptation enables the emersed animal to maintain an active, and largely aerobic metabolism over the wide range of temperatures which may be encountered.

Bivalves inhabiting the mid-littoral zone are emersed for shorter periods of time during each tidal cycle and thus need to compromise between adaptation for life as aquatic animals and the ability to survive temporary, though regular, emersions. The short term and predictable nature of tidal exposures allows the strategy of actively reducing activity to a quiescent level which can be maintained via anaerobic pathways (Helm & Trueman, 1967; Coleman, 1973; McMahon, 1988). Thus, metabolic processes can be reduced in response to emersion. Some species do have a limited air breathing capacity, although it is sufficient to maintain aerobic pathways only if the metabolic rate, and hence energy requirement, is reduced (Boyden, 1972a).

In contrast with these generalised situations, the data on sub-littoral bivalves are again rather sparse. Coleman (1976) reported that during emersion of, the predominantly sub-littoral, *Modiolus modiolus*, oxygen consumption increased up to a temperature of 16.5°C, but any further increase in temperature resulted in only a small increase in oxygen uptake. Heart rate, over the same period, showed a marked decrease, with periodic activity being interpreted as attempts to respond to the temperature increases. Consequently, oxygen consumption exhibited temperature dependence for a limited period until physiological limitations prevented greater

oxygen uptake, resulting in a gradual transition to anaerobic metabolism (Coleman, 1976). Heart rate, however, appeared to be almost completely temperature independent due to the inability of the heart to maintain effective function during emersion. A species, such as *Pecten maximus*, with no adaptation to emersion is also likely to encounter problems resulting from the basic inability of its physiological system to function in an aerial environment. Such problems will be compounded by the effects of increased activity or exposure to a higher air temperature.

In order to investigate whether the reduced survival at high temperatures, detailed in the previous chapter, was caused by the consequences of an increased metabolic rate it was necessary to determine the relationship between metabolic parameters and environmental temperature. Heart rate is considered to be a useful metabolic indicator in scallops (Bricelj & Shumway, 1991).

More importantly, perhaps, what effect does emersion at a constant temperature have on the type and rate of metabolism, particularly since adduction is such an energetic process? Since the sub-littoral *Modiolus modiolus* is capable of some aerial respiration then it might also be expected in *Pecten maximus*. However, to maintain physiological function this must be sustainable over a long emersion and be efficient enough to maintain energy production and acid-base balance.

If aerial respiration is possible then energy requirements may be sustained solely through aerobic pathways. Alternatively, if oxygen uptake is limited and metabolism is reduced then it might also be possible to supply energy via anaerobiosis, provided that the anaerobic capacity of the scallop is sufficient and it is tolerant of end-product accumulation. If the animal is active, however, or maintains an energy requirement similar to that under normal conditions, will a compromised respiratory system be adequate to sustain these needs through aerobic metabolism alone? Anaerobic pathways are almost certainly inadequate to supply sufficient energy for an active metabolism and, therefore, scallop mortality may be linked, at least in part, to the inability of the stressed physiological system to produce enough energy to maintain basic metabolic function. In addition, if the efficiency of gas exchange deteriorates in air what are the consequences for pH balance? Clearly then, an assessment of scallop respiration, during emersion could indicate which, if any, of these situations is applicable.

Another question, discussed briefly in the previous chapter, is that of metabolic waste accumulation, particularly the products of nitrogen metabolism and, if shown to be applicable, those of anaerobic metabolism. If such products are found to accumulate, as they have in some other emersed bivalves (Bayne *et al.*, 1976; Akberali *et al.*, 1977; Thompson *et al.*, 1978; Widdows *et al.*, 1979), then what are their principal effects and what bearing might they have on overall survival?

The answers to these questions would enable us to build up a picture of the nature of scallop physiology and metabolism during emersion and how it differs from the normal situation. It seems likely that some of the environmental factors investigated in the previous chapter will be the cause of any physiological changes in emersed scallops, and, if the most important ones can be identified, then potential solutions to the problem of live scallop transport may be determined and applied. In addition, an investigation of the effects that these changes have on tissue structure, extracellular fluid chemistry and cellular function may suggest further modifications to transit conditions in order to minimise these effects and so prolong the life of emersed scallops.

4.1. Heart rate response to temperature changes

Materials and method

Recordings were made according to the method described by Hoggarth and Trueman (1967). Two fine wire electrodes, with an alternating current being passed between them, were positioned close to the heart. Changes in heart volume, as a result of the heart filling and emptying, alter the electrical impedance between the electrodes and this impedance change was converted to a voltage signal via an impedance-coupler unit (Strathkelvin Instruments). The impedance unit was connected to a Washington MD2 oscillograph in order to produce a permanent record of heart rate.

Scallops were prepared for heart rate recordings by drilling two 1 mm holes on either side of the heart through the upper left valve. The mantle/pericardium was pierced and 2 shellac-coated, copper-wire electrodes (0.28mm diameter) were inserted *ca.* 5mm into the body cavity. The electrodes were cemented into place using gel-form cyanoacrylate adhesive. Care was taken not to allow adhesive or accelerator to enter the holes or shell margin.

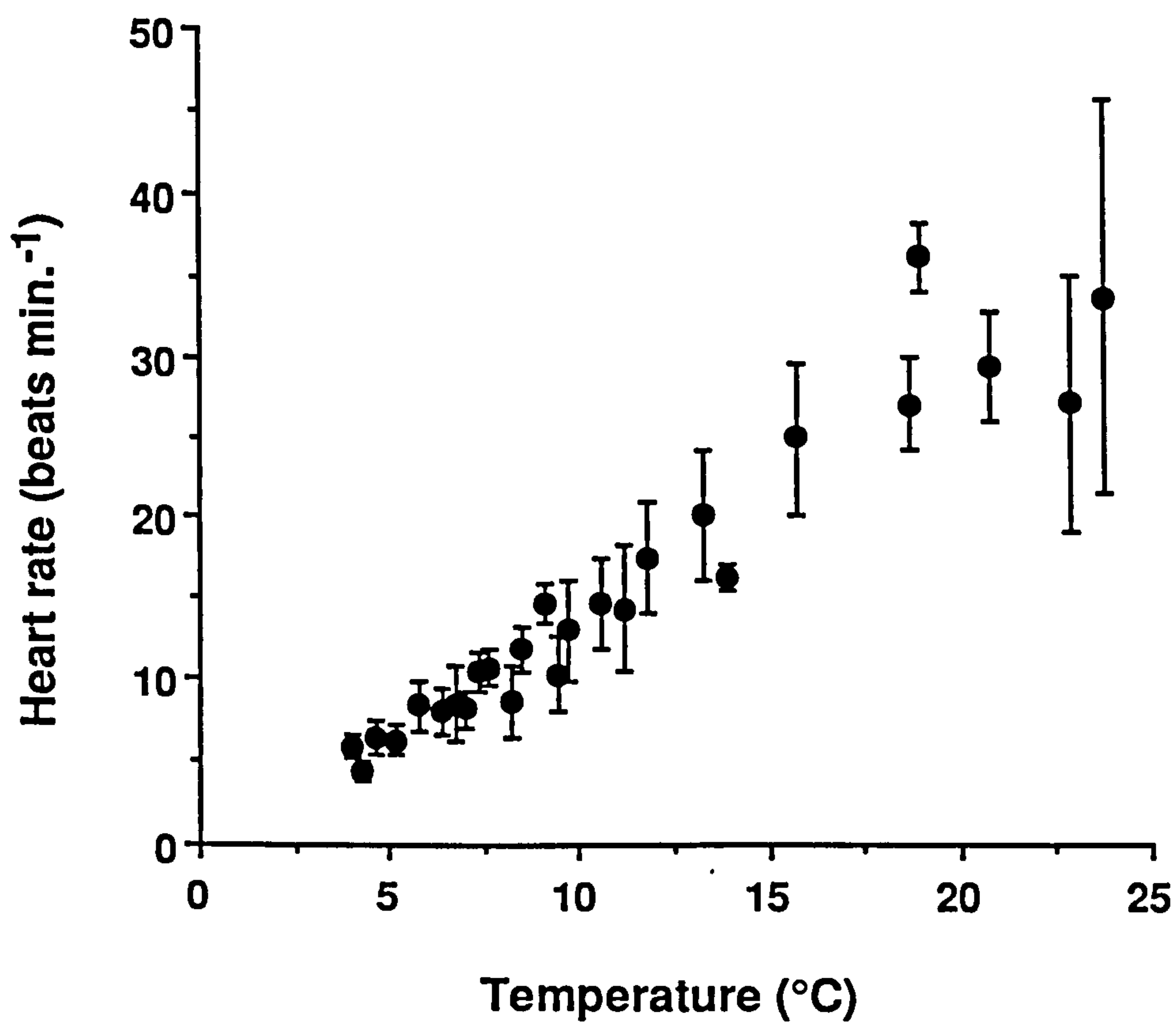
Prepared scallops were allowed to recover in a small tank of aerated sea water (10 l capacity) for 2 hours before recordings were made. The tank was fixed in a large water bath within which water temperature could be controlled using a cooling unit (Conair Churchill (Betta Tech) 02/CTC HG) and thermostatic unit (Grant KD/TD).

Heart rates of 11 animals (shell height 10 ± 1 cm) were recorded over the temperature range 4-23.8°C. Recordings were taken approximately 15 minutes after the required temperature had been reached to allow time for the physiological response to stabilise. Water temperatures were raised or lowered, over approximately 10 minute intervals, in 0.5°C increments from the initial acclimation temperature of 10°C.

Results

Heart rate exhibited a positive linear relationship with increasing water temperature between 4 and 18.7°C (test of linearity, $P=0.087$. Significance at >0.05) (Fig. 4.1). The average rate increased from 5.9 ± 0.7 beats per minute (bpm) ($n=4$) at 4°C to 27.0 ± 3.0 bpm ($n=4$) at 18.7°C, $Q_{10} = 2.8$. At higher temperatures the relationship became non-linear ($P=0.0002$) and heart rate showed no regular pattern, for example, 36.1 ± 2.13 bpm ($n=3$) at 19°C and 27.1 ± 7.9 bpm ($n=6$) at 22.9°C. Between 18.7 and 23.8°C, $Q_{10} = 1.53$.

Fig. 4.1 *Pecten maximus*. Effect of water temperature on heart rate. Values are means (\pm S.D.), n=4 to 11 at each temperature.



4.2. Measurement of aquatic respiration

Materials and method

Scallops of approximately equal size (10 ± 1 cm shell height, 54-60 g tissue wet weight), which had been kept in re-circulating aquaria at 10°C for less than 2 weeks, were cleaned of epibiota, and replaced in aerated sea water to allow the scallop to resume a respiratory water flow. This was evident by the formation of inhalant and exhalant siphons by the mantle fringe. After 30 minutes of mantle cavity ventilation, individual scallops were transferred into a perspex respirometer chamber (volume 1500 ml) fitted with a Radiometer E5046 oxygen electrode and connected to a Strathkelvin 781 oxygen meter whose output was fed to a BBC Goerz SE120 chart recorder. The chamber was placed in a water bath, at 10°C , and attached to a circulating sea-water reservoir via controllable inlet and outlet valves, thus allowing the chamber to be isolated from the circulating water supply. Sea water used in the reservoir had previously been sterilised by passing it through a UV sterilising unit for at least 60 minutes in order to reduce the effect of bacterial respiration. The oxygen electrode was calibrated using sodium sulphite in a 0.01M borax solution and aerated sea water, with both solutions at 10°C .

When the scallop was again ventilating freely, the water circulation was turned off to isolate the chamber and recording commenced. The water within the chamber was mixed using a magnetic stirring bar in order to prevent localised oxygen depletion around the electrode membrane, thus ensuring that PO_2 changes related only to respiratory demand.

Between each recording period the chamber and water circulating system were soaked in a dilute sterilising solution (sodium hypochlorite, 0.1%) for 30 minutes to prevent bacterial build up and hence keep background respiration to a minimum. The small level of background respiration, in addition to any electrode drift, was measured for a period of at least 30 minutes prior to any recording of scallop respiration and these values were subsequently subtracted from all experimental data. Recordings of scallop respiration were made for up to 1 hour, during which most animals exhibited a constant, linear rate of oxygen consumption. The oxygen concentration of fully-saturated sea water within the respirometer chamber water, was calculated via the formula:

$$\text{PO}_2 (\text{Torr/mm Hg}) = (\text{Barometric pressure (mm Hg)} - \text{Water vapour pressure (mm Hg)}) \times 0.2093$$

Given the chamber volume and the oxygen concentration of fully saturated sea water, the total oxygen content of the system could be determined. The % change over time gave scallop respiration (after correction for the background value). Following determination of tissue dry

weight, oxygen consumption could be expressed as a weight-specific rate per hour for each scallop.

Results

The mean oxygen consumption rate ($\dot{V}O_2$) of *Pecten maximus* in sea water at 10°C was $241.9 \pm 107.6 \mu\text{l O}_2 \text{ g}^{-1}(\text{dry weight}) \text{ h}^{-1}$ (n=6) (Fig. 4.2).

4.3.1. Measurement of aerial respiration

Materials and method

Scallops of $10 \pm 1\text{cm}$ shell height (54-60g tissue wet weight) were scrubbed clean of epibiota and allowed to settle prior to the experimental procedure. Each animal was then transferred to the respiration chamber of a constant pressure respirometer (Davies, 1966). The chamber was connected to a scaled manometer within which oxygen uptake was recorded as a change in fluid level. This change was converted to a volumetric change by movement of the micrometer, one division (mm) relating to $36.32 \mu\text{l}$ of gas. Carbon dioxide produced by each scallop was absorbed by filter paper rolls soaked in a 10% KOH solution and placed in holders within the chambers.

The chambers were sealed and placed in a large glass water bath (100 x 30 x 20 cm), set at $10 \pm 1^\circ\text{C}$, and allowed to equilibrate to temperature for 60 minutes. Each chamber was connected to a compressed air supply which provided air at the experimental temperature via a 3 way valve. During recording periods the valves isolated the chambers from the air supply. After equilibration, the manometers were sealed and measurements were taken every 5 or 10 minutes, depending on respiration rate, for a period of one hour.

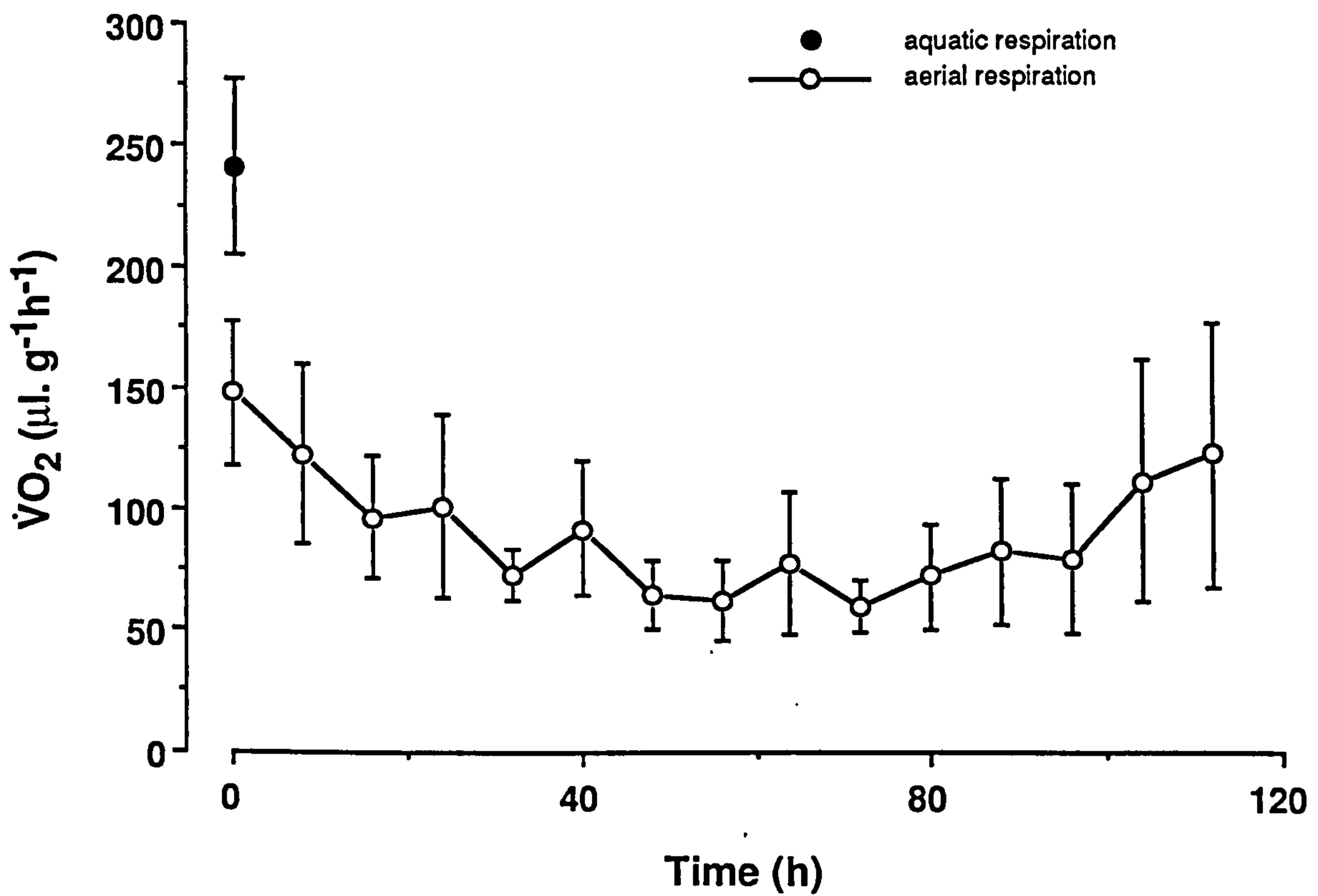
The records were made every eight hours for a period of almost 6.5 days. After the experimental recordings, the scallops were removed and the flesh dry weight was determined after oven drying at 70°C for 72 hours.

Results

The mean respiration rate of emersed *Pecten maximus* exposed at an air temperature of 10°C is shown in Fig. 4.2. The initial $\dot{V}O_2$ of $147.6 \pm 30.7 \mu\text{l O}_2 \text{ g}^{-1}$ (dry weight) h^{-1} (n=6) was significantly different from the mean aquatic rate of oxygen consumption ($P < 0.05$, 2 sample t-test). The aerial respiration rate then decreased significantly over the following 56 hours to 61.4

Fig. 4.2

Pecten maximus. Aquatic and aerial weight-specific rates of oxygen consumption at 10°C. Each value is a mean rate (n=6). Closed symbol represents the aquatic respiration rate (\pm S.D.). Open symbols represent the aerial respiration rate (\pm S.D.).



$\pm 16.6 \mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$ ($P < 0.05$, ANOVA). Figure 4.3 shows a non-parametric regression of the same oxygen consumption data. This regression, produced from the S-plus statistical software package, enables the data to describe the initial curve shape and a smoothed line is then fitted. The hatched area represents the 95% confidence interval. This new line shows more clearly that oxygen consumption remains constant (NSD, ANOVA) from 56 to 72 hours, before increasing significantly to $122.9 \pm 55.4 \mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$ after 112 hours in air. The scallops were found to be dead at this time and were therefore removed from the experimental chambers.

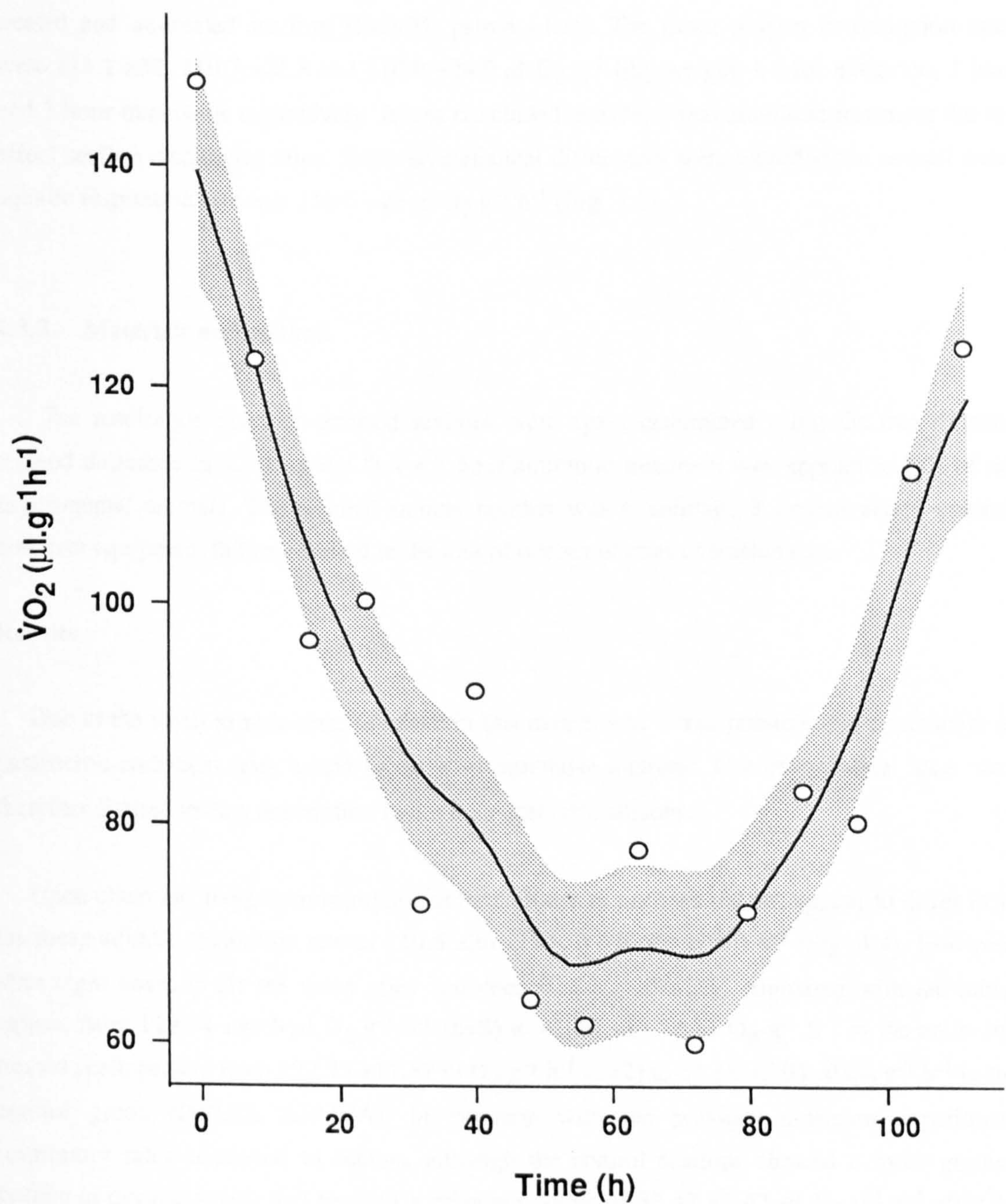
However, since the respiratory rates of the scallops had started to increase after 72 hours, when the animals were clearly in a deteriorated state, it was thought that a rapid increase in bacterial numbers might be responsible for the rise in oxygen consumption. To investigate this hypothesis further, 5ml samples of mantle cavity fluid were taken from the experimental scallops, returned to the respirometer chambers and oxygen consumption measurements resumed for the following 42 hours. At the end of this 42 hour period, the mantle fluid samples gave a mean oxygen consumption rate of $310.6 \pm 93.1 \mu\text{l O}_2 \text{ h}^{-1}$. The final consumption rate for the scallops (112 h), before correcting for weight, was $595.2 \pm 182.3 \mu\text{l O}_2 \text{ h}^{-1}$, indicating that the bacteria in the total volume of mantle fluid, considerably greater than 5ml, were the main cause of oxygen consumption in the latter part of the experiment. Microscopic examination of the fluid samples confirmed the presence of high bacterial concentrations.

Bacterial numbers capable of such significant oxygen consumption may compete with the scallop gills for available oxygen within the mantle cavity fluid and, additionally, present a complicating factor in the overall analysis of scallop aerial physiology. Such an unexpected finding therefore required further investigation, not only to determine the true nature of scallop respiration during emersion, but also to determine the effect of bacterial control on scallop survival. This latter point, and a more detailed analysis of microbiological factors, are presented elsewhere in this study, but the respiratory questions were addressed according to the following methods.

4.3.2. Materials and method

The results of the previous experiments suggested a substantial bacterial contribution to the pattern of scallop respiration in air. Pre-treatment of the experimental animals with antibiotic compounds was therefore used to determine the extent of this factor. Although the bacterial contribution to aquatic respiration rates should have been minimal, due to the precautions detailed earlier, it was important to confirm this, in addition to determining the effect of the antibiotics on the scallops themselves. The experimental method used was identical to that described in 4.2 except that the following modifications were adopted. The aquatic respiration

Fig. 4.3 *Pecten maximus*. Non-parametric regression of the aerial oxygen consumption data shown in Fig. 4.2. Fitted curve indicates periods of decreasing, constant and increasing respiration rate during emersion at 10°C. Hatched area represents 95% confidence interval of the line.



rates of 6 scallops were measured according to the method described in 4.2. The animals were then bathed for periods of 1 or 3 hours in a solution of 0.03g Benzylpenicillin sodium B.P. and 0.03g Streptomycin sulphate B.P. in 1 litre of UV treated sea water (modified from Taylor and Andrews, 1988). Respiration rates of each scallop were then measured again and results compared with the pre-treatment values.

Results

No statistically significant differences were found between the aquatic respiration rates of treated and un-treated scallops ($P < 0.05$, paired t-test). The mean oxygen consumption rates were 111.1 ± 31 , 110.7 ± 26.8 and $110.0 \pm 24.9 \mu\text{l O}_2 \text{ g}^{-1} (\text{dry weight}) \text{ h}^{-1}$ for untreated, 1 hour and 3 hour treatments respectively. It was concluded therefore, that antibiotic treatment did not affect scallop respiration rates. Since no statistical differences were recorded the overall mean aquatic respiration rate was $110.6 \pm 26 \mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$ (Fig. 4.4).

4.3.3. Materials and method

The respiration rates of emersed scallops were again determined using the experimental method described in 4.3.1 except that a 1 hour antibiotic treatment was applied to half of the experimental animals. The original sample number was 6 animals, 3 treated and 3 control, however equipment failure resulted in the loss of one set of control scallop data.

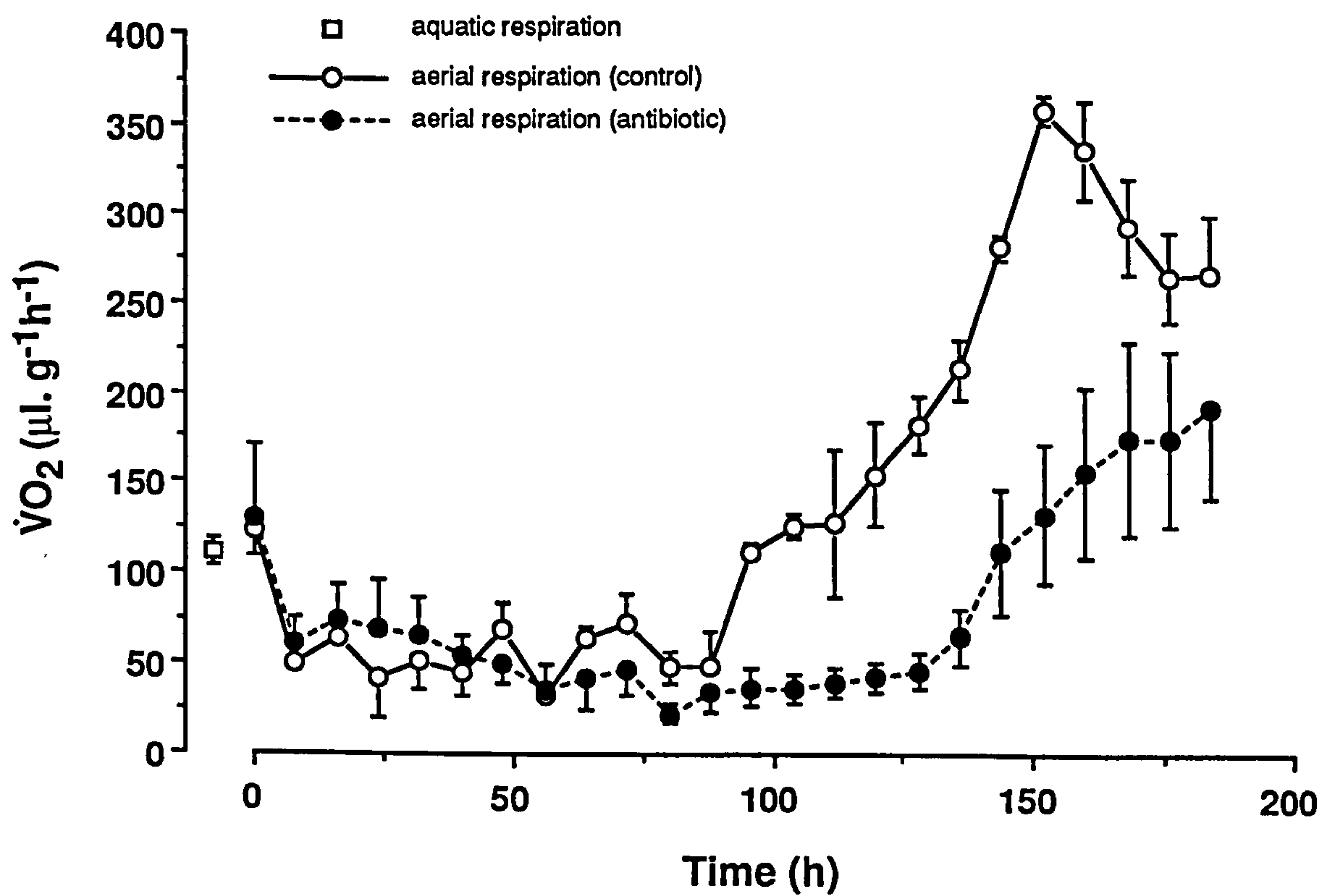
Results

Due to the small sample sizes involved in this experiment it was possible that application of parametric statistical tests would produce an unreliable analysis. Use of statistical tests were therefore limited to data description rather than data comparisons.

Upon emersion, oxygen consumption in both groups of scallops did not appear to differ from the mean aquatic respiration rate of $110.6 \pm 26 \mu\text{l O}_2 \text{ g}^{-1} (\text{dry weight}) \text{ h}^{-1}$ (Fig. 4.4). However, after eight hours in air the mean rates had decreased significantly, compared with the initial values, from $129.64 \pm 40.6 \mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$ ($n=3$) to $61.40 \pm 13.78 \mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$ in the antibiotic treated scallops, and from $122.95 \pm 11.57 \mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$ ($n=2$) to $51.18 \pm 2.01 \mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$ in the control group ($P < 0.05$, ANOVA). In common with the previous emersion experiment, respiratory rates continued to decline, although the control scallops showed a more gradual decline in oxygen uptake and reached a minimum value of $33.47 \pm 5.43 \mu\text{l O}_2 \text{ g}^{-1} \text{ h}^{-1}$ after 64 hours of exposure. This value was significantly different from the 8 hour value ($P = 0.05$, ANOVA). Thereafter, oxygen consumption increased slowly and then more rapidly, reaching

Fig. 4.4

Pecten maximus. Aquatic and aerial weight-specific rates of oxygen consumption at 10°C. All values are rates per gram of dry tissue. Square symbol represents mean aquatic rate (\pm S.D.)(n=6) of 3 runs, one without antibiotic treatment and two with antibiotic treatment for 1 and 3 hours. Treatment showed no significant differences (paired t-test). Closed symbols represent mean rates (\pm S.D.) of oxygen consumption in air by antibiotic treated scallops (n=3), open symbols represent mean rates (\pm S.D.) for untreated scallops (n=2).



357.25 \pm 8.41 μ l O₂ g⁻¹ h⁻¹ after 160 hours (P<0.05, ANOVA). Oxygen consumption values then decreased significantly over the following 32 hours (P<0.05, ANOVA) to 256.3 \pm 32.6 μ l O₂ g⁻¹ h⁻¹. The untreated scallops were found to exhibit no mantle response (see 3.3) when tested at 96 and 120 hours respectively, and must therefore be considered dead at, or shortly after, these times.

In contrast, the mean oxygen consumption rate of the antibiotic treated scallops continued to decline up to 88 hours when it was 22.87 \pm 5.1 μ l O₂ g⁻¹ h⁻¹. This value was also significantly different from the 8 hour value (P<0.05, ANOVA). After this time, oxygen consumption was found to increase to a maximum value of 190.94 \pm 51.3 μ l O₂ g⁻¹ h⁻¹ after 192 hours. This value was significantly different from that obtained at 88 hours (P<0.05, ANOVA). The 3 antibiotic treated scallops were considered dead when tested at 136, 168 and 176 hours of emersion respectively.

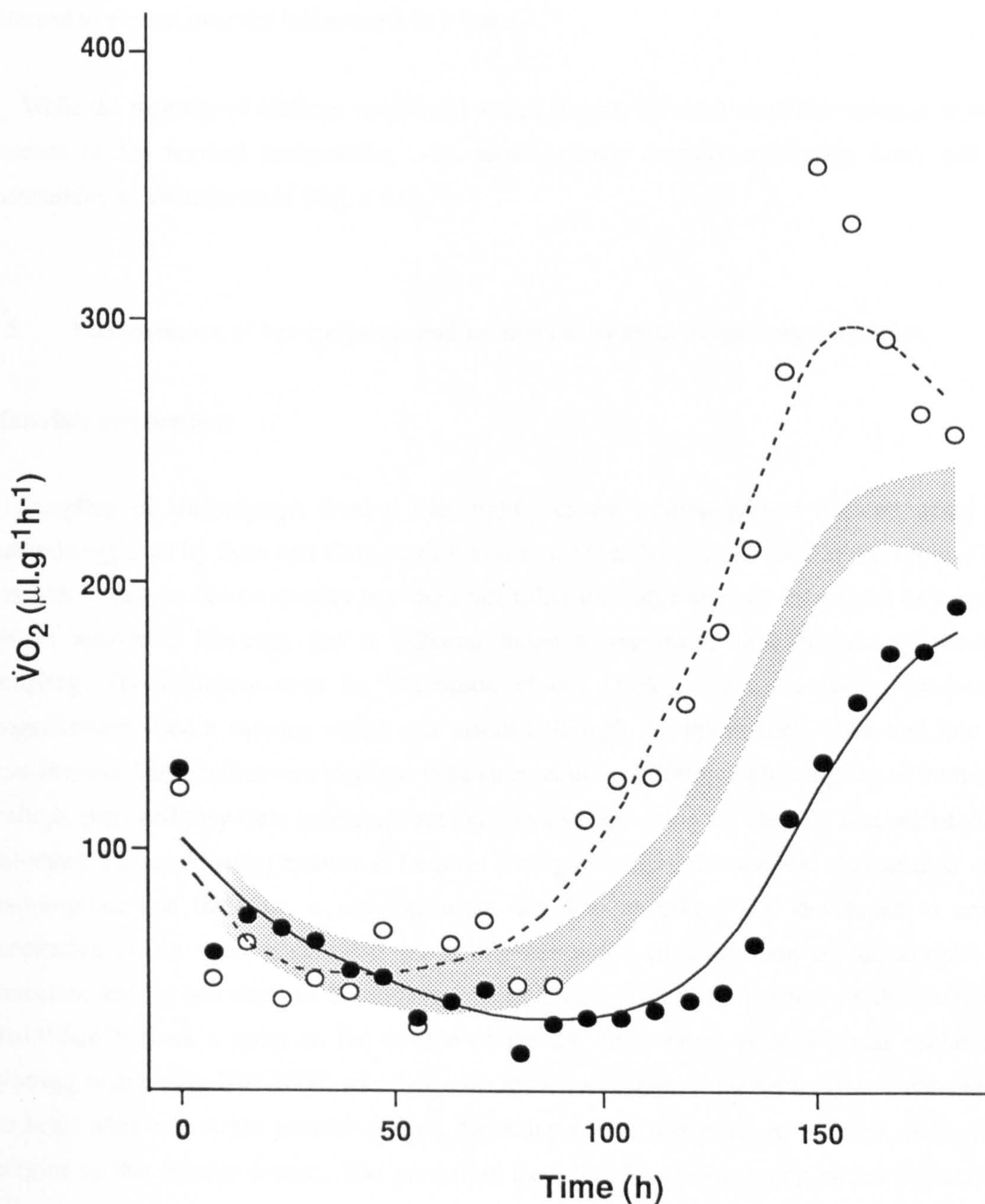
The two sets of aerial-respiration data were also analysed using the S-plus statistical package, which produced comparable non-parametric regressions (4.3.1) in order to detect the point at which the data become significantly different from each other (Fig. 4.5). Analysis using non-parametric statistics was considered to be more reliable. The hatched area is analogous to a 95% confidence band, and indicates a statistically significant difference at the point when the curves fall outside the area. The figure shows that the oxygen consumption rates for control and treated scallops were similar until shortly after the 72 hour recording. After this time, consumption rates became significantly different and remained so for the duration of the experiment.

4.4. Heart rate response to emersion

Materials and method

Heart rate recordings of scallops exposed in air (emersion) were made using the technique described in 4.1. Emersion was carried out at 10 \pm 1°C. Prepared scallops were left to settle in a small experimental tank (10 l capacity) until a regular heart rate was obtained. The water was then carefully siphoned from the tank to avoid disturbance and the cardiac responses to emersion were recorded. Following the exposure period, of approximately 4 hours, the tank was refilled and the heart rate was monitored until the pre-emersion heart rate was restored. Maintenance of heart rate recordings beyond 4 hours proved difficult using this technique, largely due to the violent adduction behaviour exhibited by this species during emersion. This activity eventually resulted in loss of the recording signal, perhaps due to displacement of the electrodes by movement of the visceral tissue.

Fig. 4.5 *Pecten maximus*. Non-parametric regression of aerial oxygen consumption data shown in Fig. 4.4. Fitted curves indicate aerial respiration rates following antibiotic treatment (full line, closed symbols) and untreated scallops (broken line, open symbols). Hatched area is a 95% confidence band and indicates that the rates become significantly different when the lines cross the band.



Results

The heart rate responses of *Pecten maximus* to emersion were variable, although a general pattern was identified (Fig. 4.6.a). During the first 10 minutes of emersion scallop heart rate was found to increase rapidly (tachycardia) by up to 20% of the initial rate. Heart rate then declined rapidly (bradycardia), and then more slowly over the following 3 hours, with some scallops maintaining a fairly constant rate of between 30 and 80% of the pre-emersion value. Upon re-immersion, a rapid tachycardia was observed in nearly all experimental animals. This increase in rate, which was frequently 20% greater than the pre-emersion value, gradually returned to normal over the following 3 to 4 hours.

While the majority of scallops conformed to this pattern the most common variation was an absence of the marked bradycardia, with some animals actually increasing heart rate or maintaining an elevated level (Fig. 4.6.b).

4.5. Measurement of haemolymph and mantle cavity fluid oxygen concentration

Materials and method

Sampling of haemolymph from a live, tightly closed scallop proved difficult using the method suggested by Fyhn and Costlow (1975) for the clam *Rangia cuneata*. In this species it is possible to insert a needle directly into the heart using the hinge teeth of the animal as a guide. *Pecten maximus*, however, has a different hinge arrangement, thus preventing accurate sampling. The technique used by Thompson *et al.*, (1980), for the scallop *Placopecten magellanicus*, used a cannula which was inserted through the upper shell valve and into the muscle sinus. This method was reported to be successful for haemolymph sampling of immersed scallops, provided they were quiescent, but during valve adduction the cannula was occasionally dislodged. Since adduction activity is frequent during emersion, this method also seemed to be inappropriate and therefore, a more accurate sampling technique was developed to ensure penetration of the heart whilst also preventing sea water contamination of the sample. By dissection and by reference to the illustrations of Dakin (1909) the position of the heart was established beneath a point on the outside of the left shell valve. A high speed dental drill (Nouvag micromotor NM 3000) was then used to etch a section of the shell (10 x 5 mm) above the heart until only slight pressure from a dissecting needle was required to break through the margins of this thinned section. The procedure took 10-15 minutes during which the scallops were wrapped in damp tissue paper to prevent desiccation. All prepared animals were allowed to recover for a full 24 hours before being used in subsequent experiments.

Fig. 4.6a *Pecten maximus*. The typical pattern of heart rate changes during emersion and subsequent reimmersion at 10°C. Lines show the responses of 3 different scallops. Time 0 indicates start of emersion, ∇ indicates reimmersion.

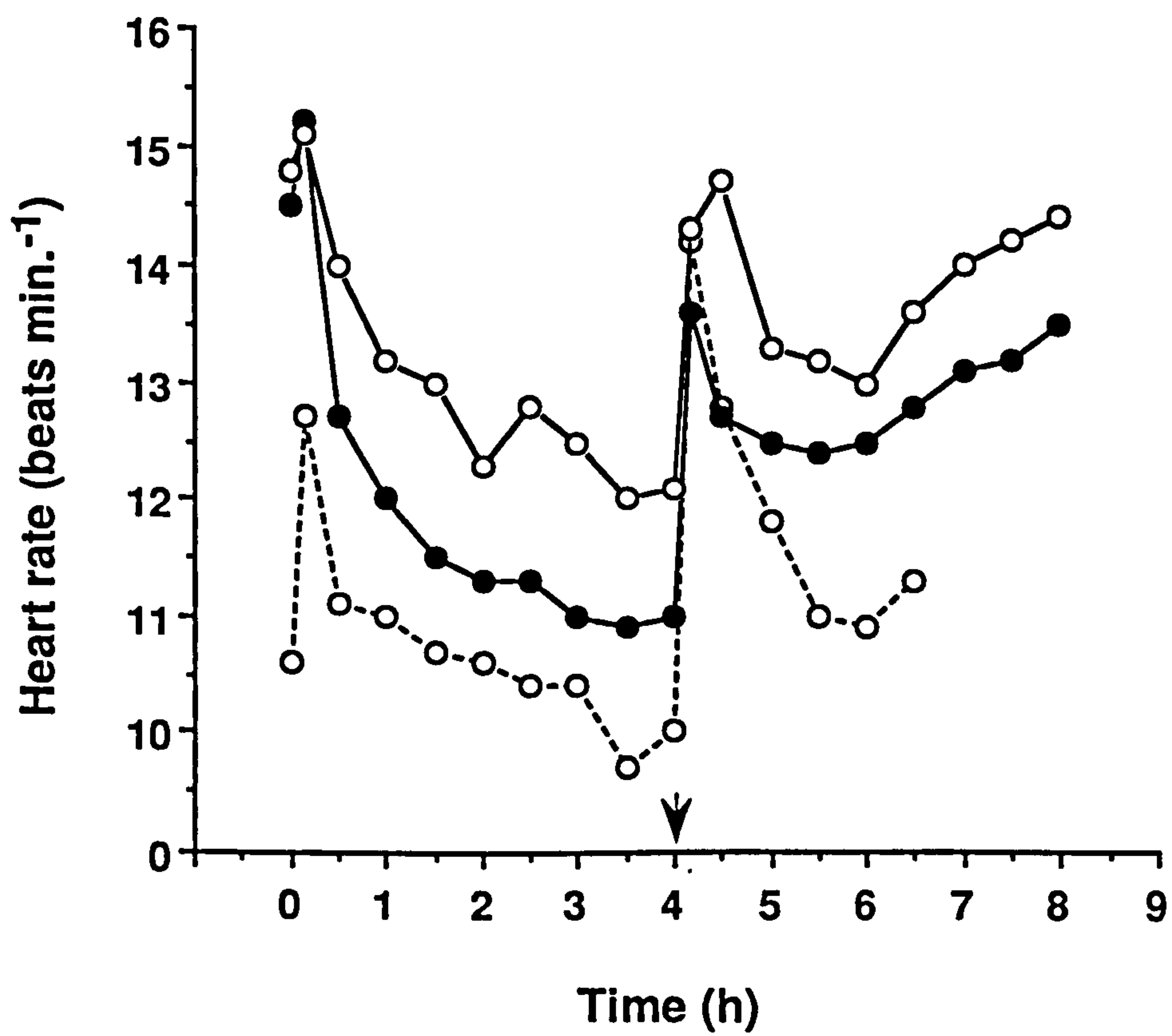
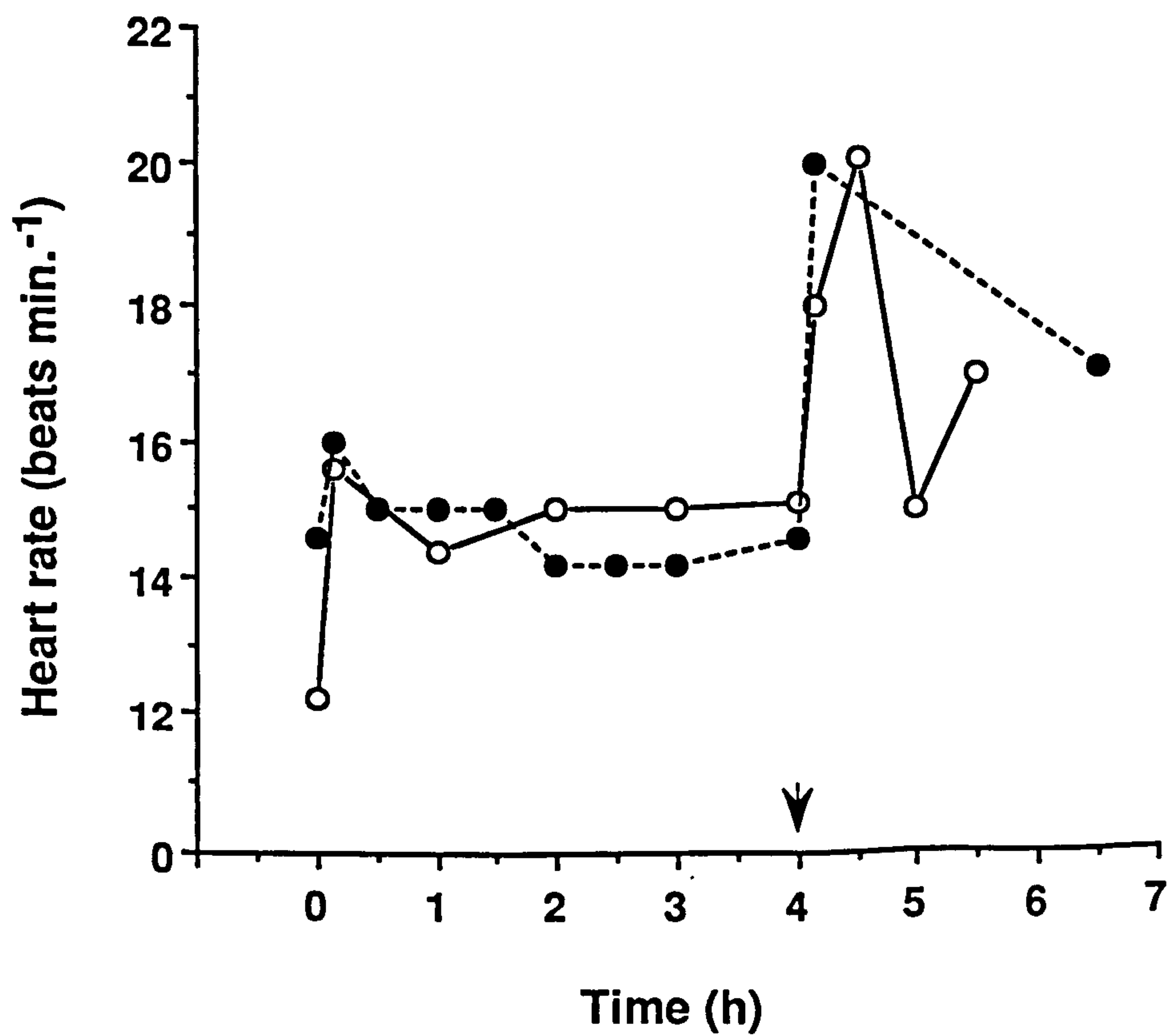


Fig. 4.6b *Pecten maximus*. Alternative pattern of heart rate changes during emersion and subsequent reimmersion at 10°C. Lines show responses of 2 individuals. Time 0 indicates start of emersion, ∇ indicates reimmersion.



Samples of 5 scallops were removed from the aquarium and emersed for 12, 24, and 48 hours at a temperature of $10 \pm 1^\circ\text{C}$ and a humidity of 95%. Another group of 5 animals was retained in the aquarium to act as a control sample. The exposures were staggered to allow measurements to be made at the same time. This experimental procedure was carried out twice to obtain sufficient sample sizes.

Sampling of haemolymph involved the removal of the shell section and cutting through the underlying mantle after which a 19 g needle, attached to a 1ml syringe, was inserted into the ventricle and 300-500 μl of haemolymph was removed. Mantle cavity fluid was obtained by inserting a needle between the shell valves and extracting a sample of approximately 500 μl . With practice this procedure could be completed within 5 seconds of the scallop being removed from the experimental tanks. The fluid samples were immediately injected into a microcell (Strathkelvin Instruments MC 100), maintained at $10 \pm 0.5^\circ\text{C}$, containing a microcathode oxygen electrode (Strathkelvin Instruments 1302) coupled to an oxygen meter (Strathkelvin Instruments 787) and chart recorder (BBC Goerz SE120). Calibration of the oxygen electrode was carried out using the method described in 4.2.

Results

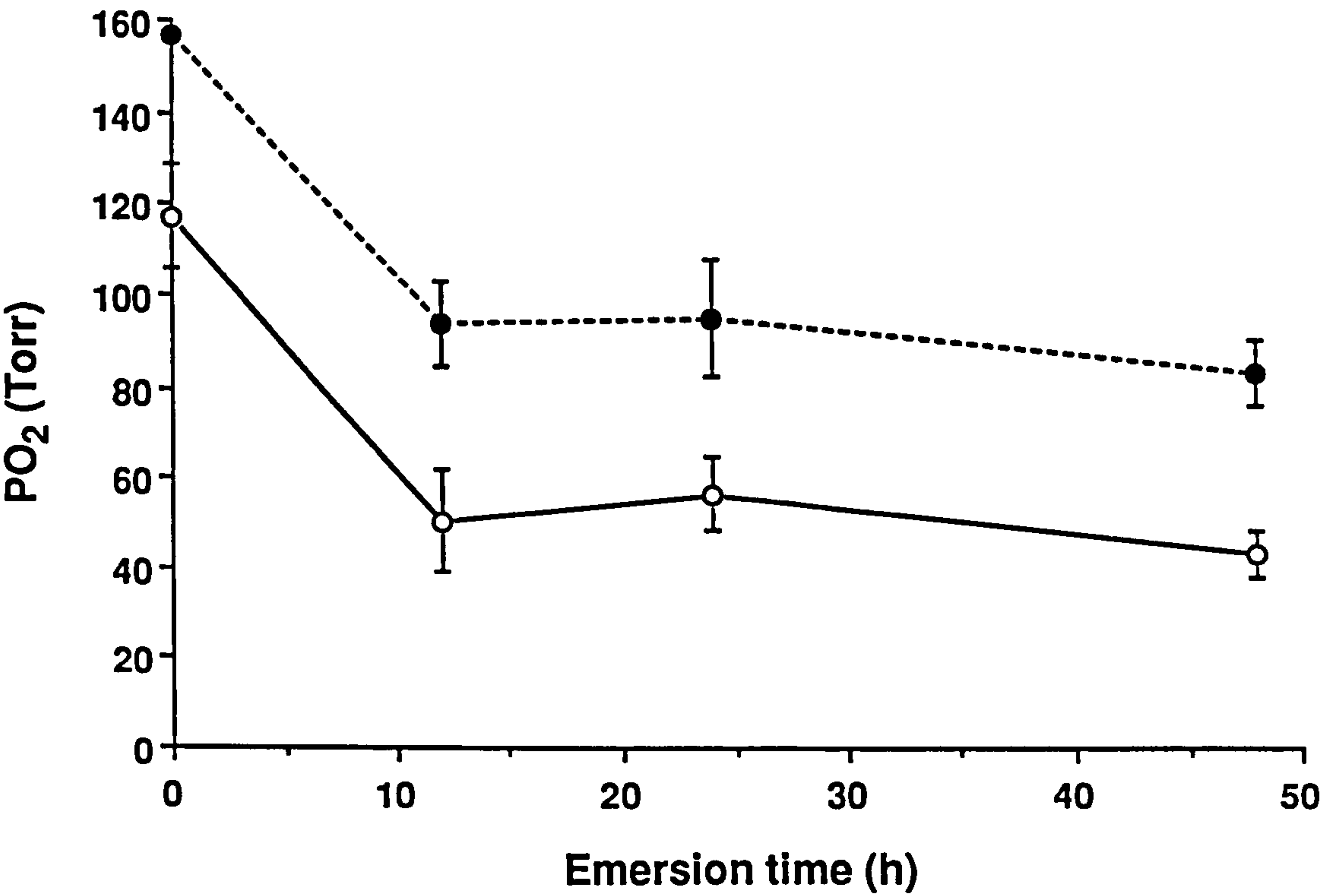
The results of both haemolymph and mantle cavity fluid PO_2 measurements during emersion are shown in Fig. 4.7. All PO_2 values were recorded as a % of fully aerated sea water at 10°C and 32‰, and converted to Torr using the equation;

$$\text{PO}_2 (\text{Torr/mm Hg}) = (\text{Barometric pressure (mm Hg)} - \text{Water vapour pressure (mm Hg)}) \times 0.2093$$

The oxygen partial pressure of mantle-cavity fluid in the control group was identical to that of fully aerated sea water, i.e. 156.9 ± 0.5 Torr. This decreased rapidly, and significantly ($P < 0.01$, ANOVA), and after 12 hours of emersion, mantle cavity fluid PO_2 was 94.12 ± 9.2 Torr ($n=4$). The oxygen partial pressure of the mantle-cavity fluid continued to decrease over the remainder of the emersion, although at a much reduced rate, and after 48 hours the value was 83.07 ± 7.4 Torr ($n=10$).

The PO_2 of haemolymph in the control scallops was 117.15 ± 11.6 Torr ($n=10$). This again decreased sharply following emersion. After 12 hours in air, the partial pressure was 50.54 ± 11.4 Torr ($n=8$) which continued to decrease, reaching 43.19 ± 5.2 Torr ($n=10$) after 48 hours. Statistical analysis (ANOVA) indicated that the initial PO_2 decrease, from 0 to 12 hours ($P < 0.01$), and the decrease from 24 to 48 hours were significant ($P < 0.01$).

Fig. 4.7 *Pecten maximus*. Changes in the PO₂ of haemolymph (open symbols) and mantle-cavity fluid (closed symbols) during emersion at 10°C. Values are means (±S.D.) (n=4 to 10).



4.6. Measurement of haemolymph and mantle cavity fluid pH

Materials and method

Experimental groups of 5 scallops were emersed at 10°C and 95 ±2% r.h. for periods of 6, 24, 48 and 72 hours. An additional group of animals was retained in fully aerated sea water at 10°C and provided control pH values. Preparation and fluid sampling methods were identical to those described in 4.5. Following removal of haemolymph and mantle cavity fluid, 80µl samples were drawn into the microcapillary pH electrode of a BMS 2 (Radiometer, Copenhagen) connected to a Corning 255 ion analyser pH meter. The electrode had been previously calibrated using Radiometer precision pH buffers.

Results

Final sample numbers ranged from 2 to 5 scallops at each time point due to difficulties in obtaining samples from the 72 hour emersion animals. The mean haemolymph pH of immersed scallops was 7.6 ±0.1 (n=4)(Fig. 4.8). Upon emersion, pH decreased rapidly, reaching 7.11 ± 0.2 (n=5) after 24 hours in air, and thereafter continued to decline over the remainder of the experimental period (ANOVA, P<0.01). By 72 hours, haemolymph pH was 6.58 ±0.3 (n=2).

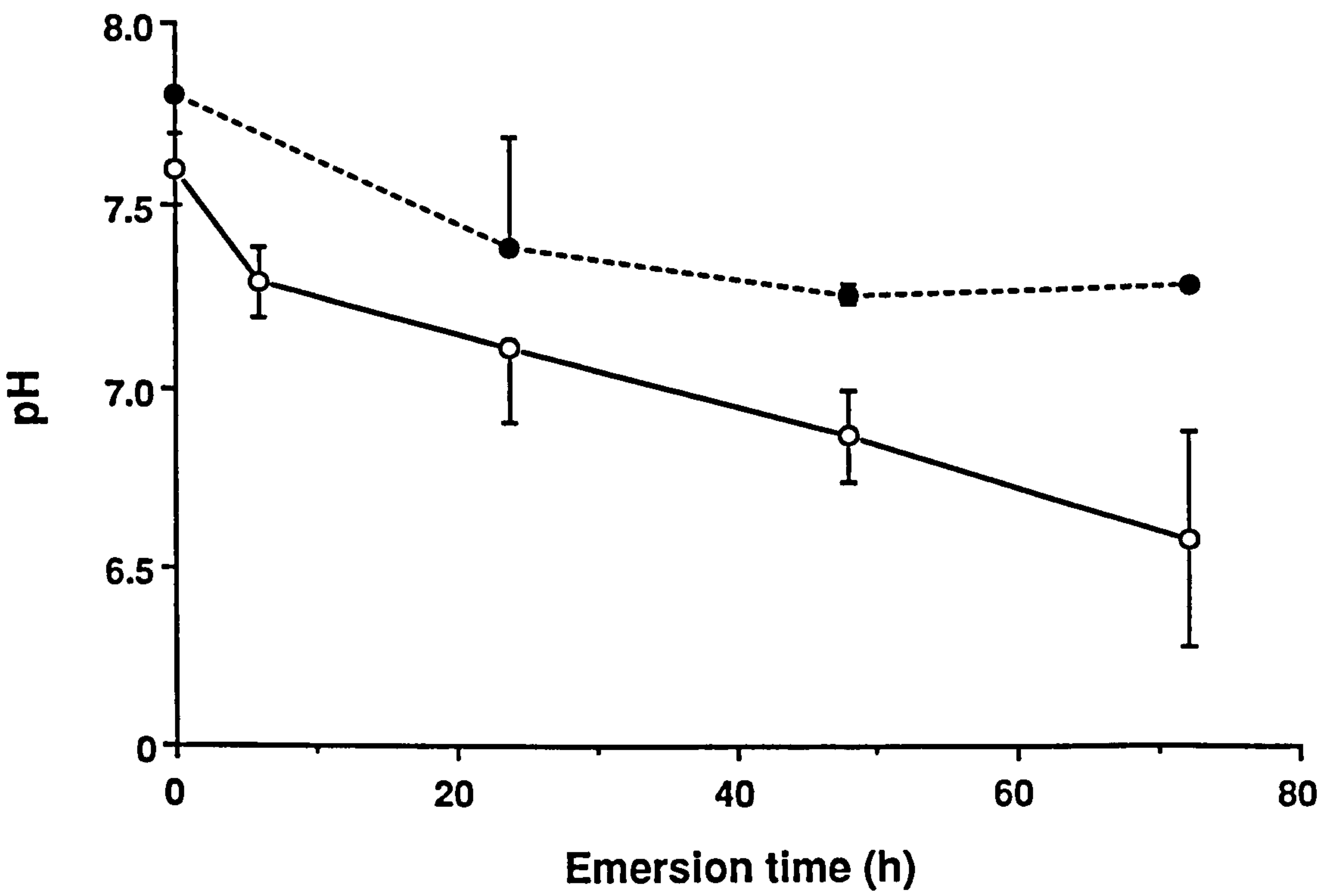
The pH of scallop mantle cavity fluid exhibited less dramatic changes during the experimental emersion (Fig. 4.8). The pH of mantle fluid from the control scallop group, i.e. that of sea water, was 7.8. During emersion for 72 hours, however, mantle-cavity fluid pH decreased significantly to 7.29 ±0.01 (n=2)(ANOVA, P<0.01).

4.7. Measurement of haemolymph and mantle cavity fluid ammonia concentrations

Materials and method

Scallops were prepared for haemolymph sampling as described in section 4.5. Following a settlement period of 24 hours after preparation, 20 animals were removed from the aquarium and divided into 4 groups of 5 scallops. The groups were then emersed for 6, 12, 24 and 48 hours respectively at a temperature of 10 ±1°C and a relative humidity of 95 ±2%. A further sample of scallops were retained in aerated, recirculating sea water to provide a control group value for haemolymph ammonia.

Fig. 4.8 *Pecten maximus*. Changes in the pH of haemolymph (open symbols) and mantle-cavity fluid (closed symbols) during emersion at 10°C. Values are means (\pm S.D.) (n=2 to 5). One set of error bars have been omitted from the 24 hour values for clarity.



After emersion for the appropriate period, a 19g needle was inserted into the heart (ventricle) and a haemolymph sample of 300-500 μl drawn into a 1ml syringe, as described in 4.5. At the same time an equivalent volume of fluid was taken from the mantle cavity.

The method used to determine the concentration of ammonia was based on the Berthelot reaction and has been modified from the method described by Liddicoat *et al.* (1975). The reaction involves the formation of chloramine which reacts with phenol in the presence of a catalyst to form quinonechloramine. This compound, in turn, reacts with phenol to produce indophenol which, at a high pH, dissociates to produce a blue colouration, the absorbance of which can be measured.

The use of sodium citrate in the oxidising solution prevents the precipitation of Mg^{2+} and Ca^{2+} from the sea water at the high pH used, by complexing the ions with citrate (Solorzano, 1969). Ammonia and other nitrogenous compounds are common contaminants of water, glassware and skin and it was necessary to use the cleanest available water, either double distilled (d.d.) or d.d. and deionised. Glassware was washed thoroughly in Decon 90 detergent, rinsed in deionised water three times and soaked in 15% Hydrochloric acid for 2 hours. Finally, the glassware was rinsed three times in d.d., deionised water and dried in a drying cabinet. Following cleaning, the glassware was handled only when gloves were being worn.

The samples of haemolymph and mantle fluid were diluted with artificial sea water, which constituted 31g sodium chloride, 10g magnesium sulphate and 0.02g sodium bicarbonate in 1 litre of distilled water, to produce 1 ml samples to which was added 40 μl of a phenol-alcohol solution (5g phenol in 50 ml of 95% ethyl alcohol), 100 μl of oxidising solution (20g trisodium citrate in 40 ml of distilled water to which, 0.2g sodium dichloroisocyanurate was added and the solution made up to 100ml with distilled water), and 40 μl of a catalyst (0.5g potassium ferrocyanide in 100 ml d. water, stored in an amber bottle).

Each sample was shaken between additions and finally vortex-mixed before being exposed to ultraviolet radiation (wavelength = 254nm) for 60 minutes. The exact timing may vary for a noticeable blue colour to develop, but this variation is controlled for by using ammonium sulphate standards. Ammonium sulphate was dissolved in artificial sea water (as above) to produce standards of 2, 4, 8, 16 and 32 $\mu\text{mol. NH}_4\text{-N.l}^{-1}$.

The absorbance of each sample was read at 640 nm in 1 cm semi-micro cuvettes using a Philips PU 8620 UV/Vis spectrophotometer and the ammonia concentration determined by relating these values to a standard calibration curve. The spectrophotometer was zeroed using a distilled water blank before each group of 5 readings. The ammonia concentration of each sample was corrected to take account of dilution factors.

Results

The results presented are pooled data from two separate analyses. Changes in the ammonia concentration of haemolymph and mantle cavity fluid drawn during emersion are shown in Fig. 4.9 a and b respectively. The mean haemolymph ammonia concentration of scallops maintained in aerated sea water was $17.1 \pm 19.7 \mu\text{mol. l}^{-1}$ (n=6). During emersion, the mean haemolymph ammonia concentration increased linearly from $73.4 \pm 135.1 \mu\text{mol. l}^{-1}$ (n=8) at 6 hours to $448.6 \pm 221.7 \mu\text{mol. l}^{-1}$ (n=7) after 48 hours. A test of linearity gave a P value of 0.82, where $P > 0.05$ is a significant linear relationship. The recorded concentration increase from 0 to 48 hours was statistically significant ($P < 0.05$, ANOVA).

The ammonia concentration of mantle cavity fluid in immersed scallops was equal to that of the surrounding aquarium sea water and since this sea water value was subtracted from all sample measurements as a background figure, the control value for mantle fluid had an ammonia concentration of zero. Actual mantle fluid ammonia concentrations also showed a significant and linear increase over the emersion period ($P < 0.05$, ANOVA)(Linearity test, $P = 0.98$). The mean 6 hour value was $91.2 \pm 136.7 \mu\text{mol. l}^{-1}$ (n=8), which increased to $413.6 \pm 168.3 \mu\text{mol. l}^{-1}$ (n=7) after 48 hours in air.

4.8. Ammonia toxicity (96 hour mean lethal tolerance)

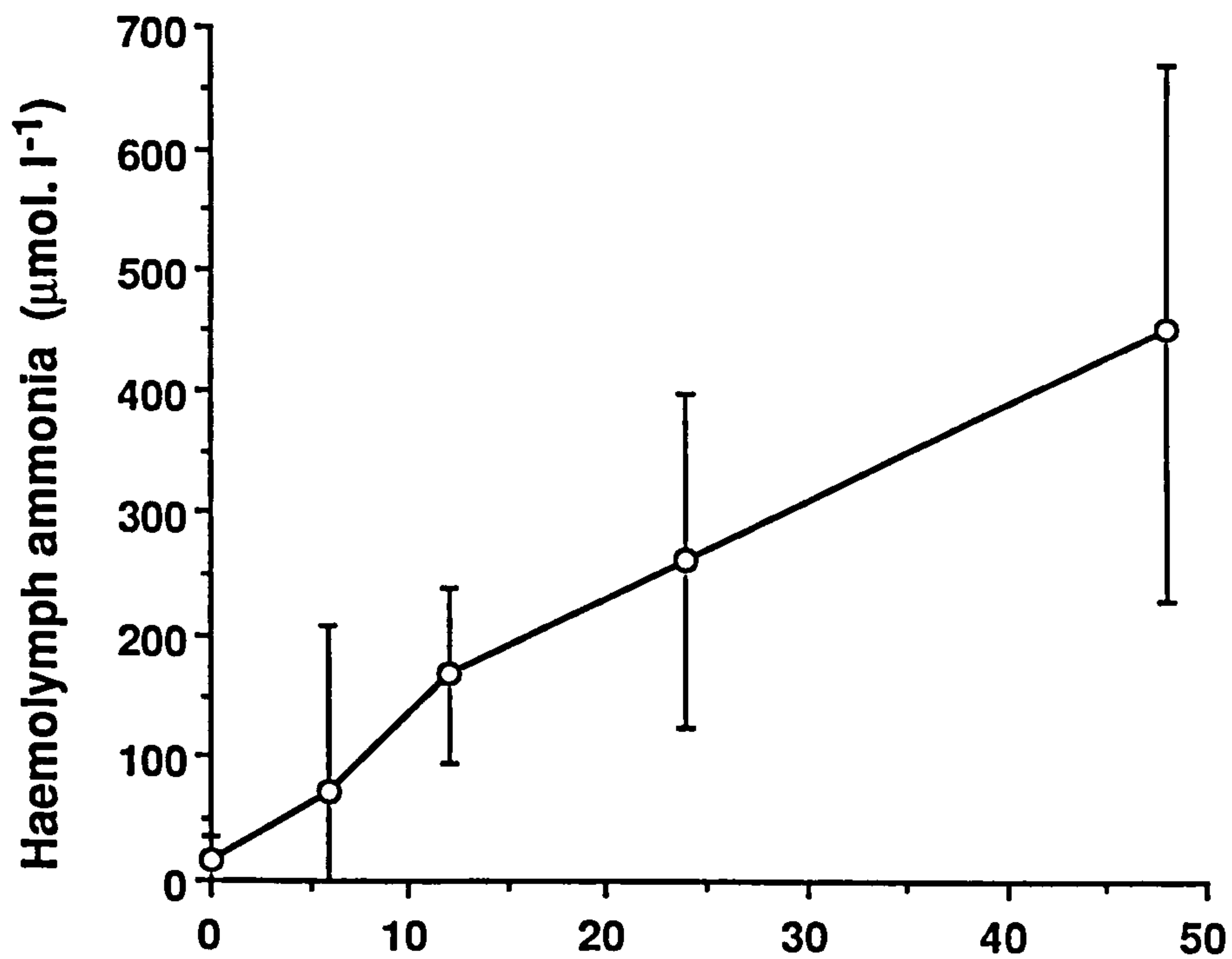
Materials and method

The toxicity of ammonia to *Pecten maximus* was assessed over a 96 hour exposure period, between the 18th and 22nd of August, 1992, at the Sea Fish Industry Authority marine farming unit, Argyll, Scotland. The procedure used was modified from the method described by Epifanio and Srna (1975). The term ammonia is used to describe both ionised (NH_4^+) and un-ionised (NH_3), the proportions of which are determined primarily by the pH of the solution, with more NH_3 occurring in more basic solutions.

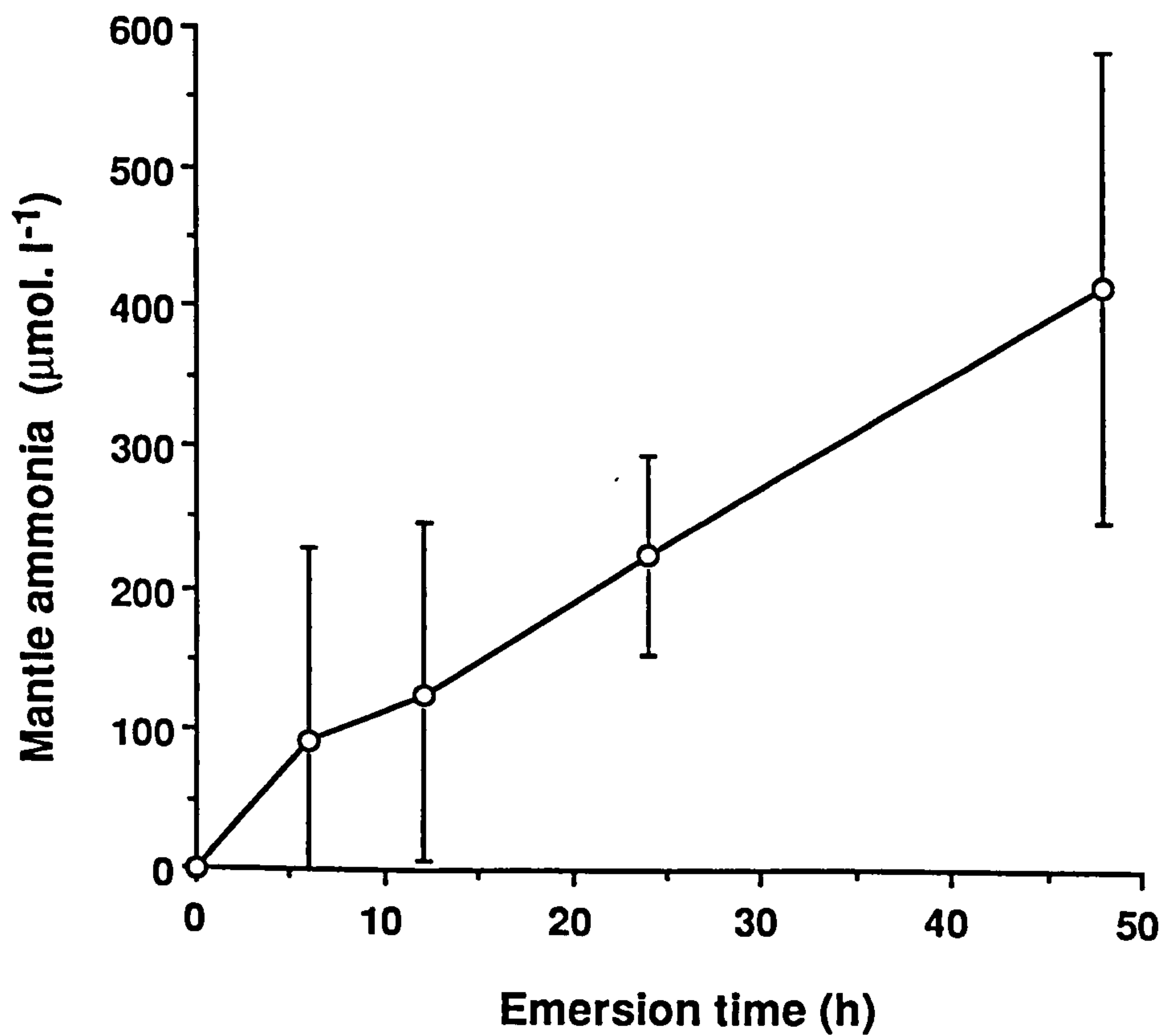
Twelve polypropylene tanks (71.5 x 11.5 x 10cm, volume = 8.2 litres) were connected to a sea-water header tank which delivered 200ml of water per min. to each tank. The water temperature was $13.5 \pm 1^\circ\text{C}$ with a salinity of $33 \pm 1\text{‰}$. The header tank was constantly filled by sea water pumped from Kentra Bay, 200m from the research unit. Each tank was connected to an air supply which provided gentle aeration, sufficient to maintain an oxygen concentration of $99 \pm 2\%$. Sea water pH ranged from 8.04 to 8.22 during the experimental period.

Figs. 4.9a, b *Pecten maximus*. Changes in the total ammonia concentration of haemolymph and mantle-cavity fluid during emersion at 10°C and 95% r.h. Values are means (\pm S.D.) (n=6 to 11).

a



b



Two year old *Pecten maximus* were cleaned of large fouling organisms prior to transferring 20 animals to each experimental tank. Scallop shell height, which was also measured at this time, ranged from 55 to 72mm.

Stock solutions of ammonium chloride (NH_4Cl) were prepared by dissolving reagent grade NH_4Cl in UV treated, filtered sea water ($0.5\mu\text{m}$ cartridge filter). The stock solution was made up in 10 l volumes and drawn through a range of 5 tubing diameters via a 10 channel peristaltic pump (Autoclade model VL). Tubing bore diameters of 0.51, 1.01, 1.52, 2.03 and 2.54 mm were used in duplicate and connected at the distal ends to lengths of tubing which fed the stock solution directly into the sea water supply of each experimental tank. A 250mM stock solution of ammonia (i.e. 741mM NH_4Cl) was passed through the pump and the flow of each channel was individually calibrated. The flow of stock solution was diluted by the sea water input of each tank (200ml. min^{-1}) to produce the final ammonia concentration of the water inlet and hence the ammonia concentration of the tank itself. Each tank was fitted with an overflow which was directed to waste.

The expected tank concentrations, taking into account pump speed, tubing bore and sea-water dilutions, were 3210, 3065, 2188, 2143, 1260, 1228, 533, 503, 70 and $58\mu\text{mol.l}^{-1}$ ammonia. Two control tanks, without an ammonia input, were also included, accounting for the total of 12 experimental tanks. Pump channel and experimental tank number were connected in a random manner to eliminate any effects of tank position within the room. To prevent potential contamination from high concentration tanks to low concentration tanks, e.g. from aeration or valve adduction, polythene barriers were erected between all experimental tanks.

The ammonia concentration of each tank was tested daily during the first two days, and twice daily on days 3 and 4. The method for ammonia analysis was identical to that described in section 4.7, except that a Camlab DR2000 spectrophotometer was used to read sample absorbance. Water temperature, salinity and pH were recorded twice daily.

The criteria of death was gaping of the valves and lack of response to mechanical stimulation of the mantle edge. Observation of the scallops continued throughout the trial and after 80 hours a number of animals were considered to be dead and therefore removed from the trial. This was done to prevent deterioration of the scallop tissue which may have affected ammonia concentrations or led to the death of other scallops through water contamination.

After 96 hours of exposure to the experimental concentrations surviving individuals were transferred to normal sea water for 36 hours before being returned to offshore lantern nets. These individuals were assessed after approximately 1 month at sea and used as a guide to the effect of sub-lethal ammonia concentrations on re-laid aquaculture stock. The number of

scallops surviving after the 96 hour experimental period was used as the basis for the 96 hour lethal tolerance limit.

Results

The results of this trial, including an assessment of sub-lethal effects are shown in Table 4.1.

After 96 hours exposure to the highest ammonia concentration of 3136 $\mu\text{mol.l}^{-1}$ (mean of two equivalent tanks) all experimental scallops had died. Scallops exposed to a mean concentration of 2165 $\mu\text{mol.l}^{-1}$ showed 72.5% mortality after the experimental period. Mortality then decreased further with decreasing ammonia concentration to only 2.5% after a 96 hour exposure to a concentration of 518 $\mu\text{mol.l}^{-1}$. No scallop mortalities were recorded during the 96 hour experimental period in tanks with an ammonia concentration of 64 $\mu\text{mol.l}^{-1}$ or in the sea water controls.

The acute toxicity of ammonia, defined here as the 96 hour median tolerance limit (TLm), was determined from the results shown in Table 4.1 after logistic transformation of the % mortality data ($\log (\% / 100-\%)$). Transformed data were plotted against log ammonia concentration. The regression equation obtained was based on the ammonia concentrations ranging from 2165.5 to 518 $\mu\text{mol.l}^{-1}$, since extremes of the range tend to be less accurate and values of 0 and 100% do not transform in this instance. The TLm value was recorded at the intercept on the x-axis (Epifanio & Srna, 1975), in this case 1858 $\mu\text{mol.l}^{-1}$ ammonia.

Table 4.1 Acute and sub-lethal toxicity of ammonia to the scallop *Pecten maximus*. Ammonia concentrations are based on the mean of duplicate experimental tanks each containing 20 scallops. Recovery was in circulating aerated sea water and sub-lethal toxicity was determined after 40 days in offshore lantern nets.

Mean ammonia concentration $\mu\text{mol. l}^{-1}$	% Mortality at 96 hours (acute toxicity)	% Mortality after 36 hours recovery	% Mortality after 40 days (sub lethal toxicity)
3136.5	100	100	/
2165.5	72.5	97.5	100
1244	12.5	25	80
518	2.5	2.5	37.5
64	0	2.5	5
sea-water control	0	0	2.5

A measure of the sub-lethal toxicity of ammonia was determined by monitoring mortality in the experimental scallops over the 40 day period following the end of the trial. At the end of the 36 hour recovery period, mortality ranged from 97.5% in the 2165 $\mu\text{mol.l}^{-1}$ sample to 2.5% in the 64 $\mu\text{mol.l}^{-1}$ sample. Mortality was unchanged in the 518 $\mu\text{mol.l}^{-1}$ and control groups. Forty days after the end of the trial, scallop mortality had reached 100% in the 2165 $\mu\text{mol.l}^{-1}$ sample, 80% in the 1244 $\mu\text{mol.l}^{-1}$ sample, 37.5% in the 518 $\mu\text{mol.l}^{-1}$ sample, 5% in the 64 $\mu\text{mol.l}^{-1}$ sample and 2.5% in the control group.

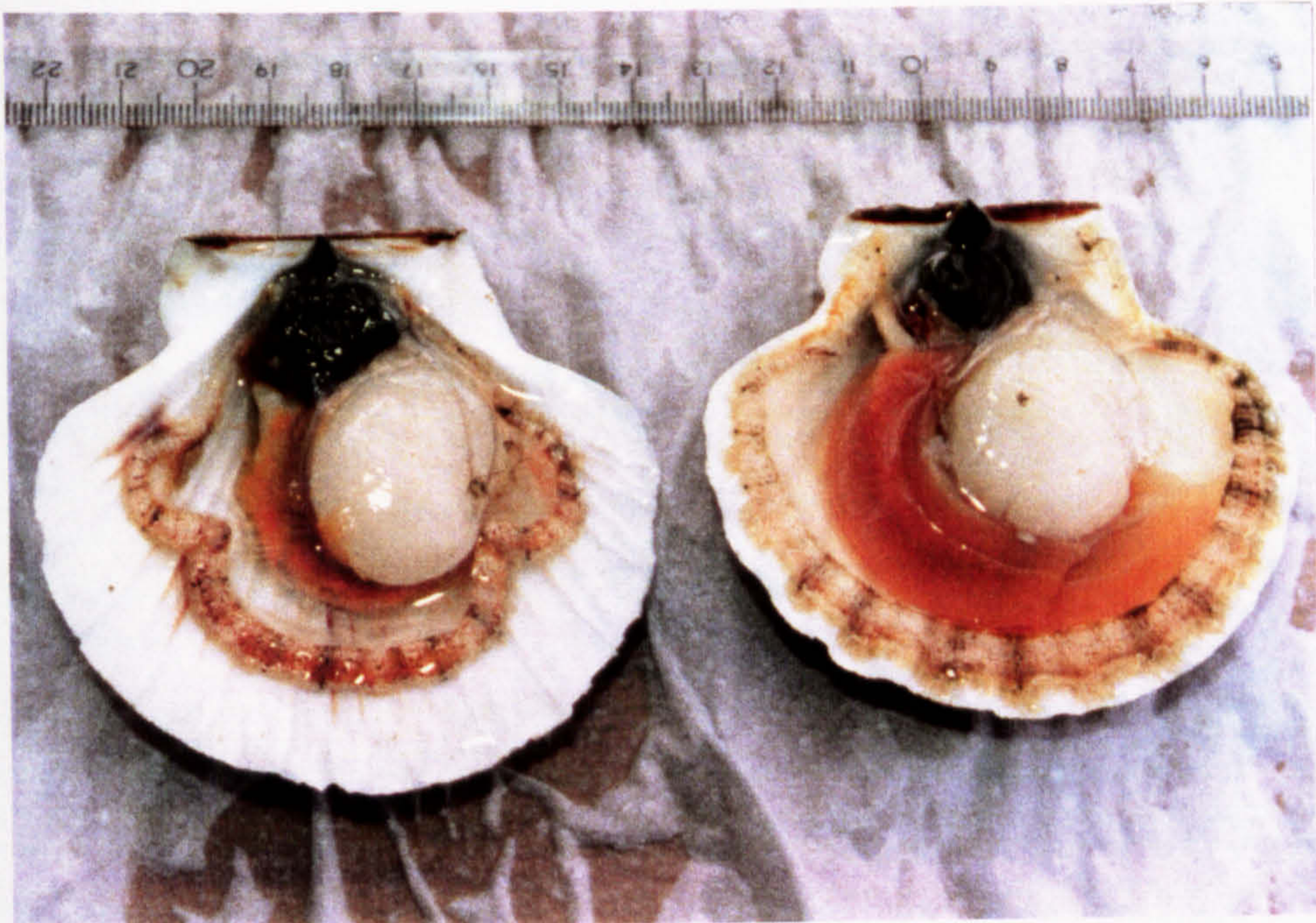
The ammonia assays, which were carried out to confirm predicted tank concentrations, gave results within 85% of the estimates for the 3 highest concentration tanks, i.e. 3136, 2165 and 1244 $\mu\text{mol.l}^{-1}$ and within 78.5% of the 518 $\mu\text{mol.l}^{-1}$ estimate. However, sensitivity of the assay proved to be much lower for the 64 $\mu\text{mol.l}^{-1}$ concentration which was believed to relate to a number of factors. The sensitivity of the assay, which from previous experiments indicated loss of accuracy below 5 $\mu\text{mol.l}^{-1}$, may have been poorer than this due to the limitations of the available spectrophotometer. This was probably compounded by the practice of sample dilution which would tend to give readings in the lower, and hence less accurate, region of the calibration curve. The effect of this would be that later adjustment by the relevant dilution factor would significantly underestimate the final value. Examination of the assay data, mortality results and the relative performance of a higher quality spectrophotometer (Philips PU8620 UV/Vis) has lead me to believe that the actual ammonia concentrations of the 64 $\mu\text{mol.l}^{-1}$ tanks were closer to the desired value than the assay had indicated, and as such the determined mortality results from these tanks were considered to be valid.

4.9. Scanning electron microscopy of gill tissue

During emersion experiments and the ammonia toxicity trial it was observed that the gills of *Pecten maximus* were particularly susceptible to deterioration. In both cases the outer (ventral) edge of the gill became frayed and eroded, before gradually separating into individual filaments. Plate 4.1 allows comparison of a scallop exposed to a high ammonia concentration (3136 $\mu\text{mol.l}^{-1}$) for 3 days and a control animal. The effects on the gills are pronounced, with few individual filaments visible in the ammonia-exposed scallop. Although this example relates to acute ammonia exposure, similar effects were observed in emersed animals over a longer time period and since ammonia had been shown to accumulate in both haemolymph and mantle-cavity fluid it was decided to investigate the structural changes which occurred in gill tissue during emersion in air.

Plate 4.1

Pecten maximus. The internal appearance of a scallop following a 72 hour immersion in sea water with an ammonia concentration of $3136 \mu\text{mol.l}^{-1}$ (left-hand specimen). Right-hand specimen shows control scallop, immersed for the same period in normal sea water. Note retracted mantle edge and eroded gill filaments of the experimental animal.



Materials and method

Specimen preparation

Fifteen scallops were exposed to air at $10 \pm 1^\circ\text{C}$ and $95 \pm 2\%$ r.h. After emersion for 24, 48, 72 and 96 hours the left (upper) gill was excised and sections, of approximately 5×10 mm, running dorso-ventrally, were cut from it. Gill sections from a control group which had been retained in water were dissected at the same time as the exposed scallops.

The sections were then placed in 2 dram vials and fixed in a solution containing 8 ml of 25% glutaraldehyde (Sigma chemicals, G5882), 10 ml of 1M sodium cacodylate, 5ml of 1M NaCl and 0.05ml of 1M CaCl_2 in 50ml filtered sea-water buffer (made up to 100ml with distilled water, pH 7.6). Sections were left in the fixative for 2 hours at a temperature of 4°C . The tissue was then given 3, five minute rinses in 0.1 M sodium cacodylate / filtered sea water buffer (as above but lacking glutaraldehyde) in order to remove all traces of glutaraldehyde. The fixed tissue was stored in buffer overnight at 4°C . After fixing and rinsing, the buffer was drained to the level of the tissue and made up with an equal volume of 2% osmium tetroxide, resulting in a 1% solution overall. The tissue was left for 1 hour at room temperature prior to three, 10 minute rinses with distilled water.

A further fixation with 0.5% uranyl acetate was carried out in the dark for 1 hour and was followed by three washes with distilled water. The fixed gill tissue was then dehydrated through graded acetone of 30, 50, 70, 90%, absolute and 'dried' absolute (absolute acetone containing water absorbing compound, 3A molecular sieve, BDH chemicals Ltd.). Each grade required 3 changes during a 15 minute period and care was taken to prevent exposure of the tissue which may have resulted in tissue damage due to solvent evaporation. Dehydration was then completed in a critical point dryer using liquid CO_2 as the transitional fluid. After this stage the specimens were stable and could be stored in a desiccator at room temperature.

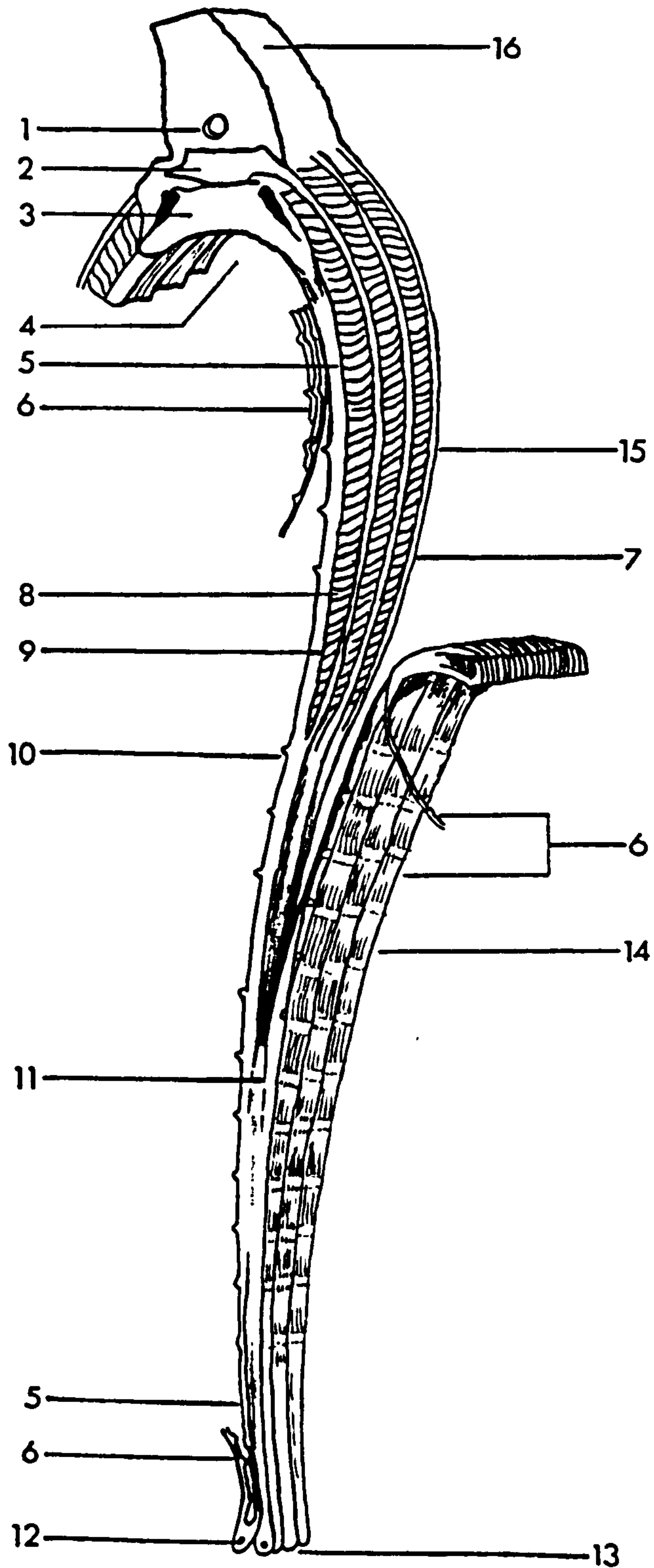
Finally, the gill tissue sections were mounted on aluminium stubs, using quick drying silver paint, and coated with a gold/palladium alloy in a Polaron E5000 sputter coating unit. Specimens were viewed using a Philips 500 scanning electron microscope (S.E.M.) (3 Kv) and photographed with a Super Rollex oscillophot M4.

Results

The general structure and principal reference features of the scallop gill are illustrated in Figure 4.10 (redrawn from Beninger *et al.*, 1988).

Fig. 4.10

General view of scallop gill illustrating the main structural features referred to in text and micrograph descriptions. 1- branchial nerve, 2- afferent branchial vessel, 3- efferent branchial vessel, 4- gill arch, 5- lateral wall of principal filament, 6- ordinary filaments, 7- afferent vessel, 8- interconnecting vessel, 9- efferent vessel in principal filament, 10- ciliated spur (cilifer), 11- interlamellar junction, 12- ciliated disc, 13- ventral bend, 14- ascending tract, 15- descending tract, 16- gill axis. Redrawn from Beninger *et al.* (1988).



Unexposed gill (Plate 4.2) The area shown is the outer surface of the ascending filaments, and the junction between the descending and ascending filaments, or ventral bend (VB), can be seen in the lower portion of the plate. Principal filaments (PF) are separated by bundles of 10-20 ordinary filaments (OF) which are densely ciliated with frontal and latero-frontal cilia on their frontal surfaces. These cilia types are involved in particle carriage and generation of respiratory currents. Ordinary filaments are joined to each other by means of ciliated spurs called cilifers (c)(Plate 4.3), which are found on the abfrontal surface of the filaments. These cilifers have ciliated discs (cd) which link with the adjacent spurs, rather like hair brushes, and thus maintain the regular surface and structural integrity of the gill as shown in plate 4.2. Gills in which successive filaments are joined by ciliated discs are termed filibranch, specifically in this case, an euleutherorhabdic plicate gill, since the two filament types are suspended from the gill axis in a plicate manner (Beninger & Le Pennec, 1991). The other main gill type found in bivalves, the eulamellibranch, has filament connections composed of vascular tissue. The structural significance of these different gill forms will be discussed.

Emersed gill (Plate 4.4). After 24 hours in air the ascending filament structure remains largely intact, although small gaps do appear where ordinary filament junctions have started to break down, suggesting initial deterioration of the tissue junctions. This plate also shows the principal filaments (PF) which separate the bundles of ordinary filaments, and are responsible for gill support, particle transport and expansion/contraction of the gill surface (Beninger & Le Pennec, 1991). In the upper right corner, where some ordinary filaments have separated, the underlying afferent (AV) and interconnecting branchial blood vessels (IV) are visible.

Emersed gill (Plate 4.5). This plate shows the ascending tract after 48 hours of emersion. Breakdown of the cilifer connections between ordinary filaments is more pronounced and strands of mucus (m) are visible on the surface, possibly produced in response to the unfavourable chemical conditions or the activity of microorganisms. Also visible at the top left are the abfrontal surfaces and cilifers (c) of the ordinary filaments comprising the descending tract.

Emersed gill (Plate 4.6). After 72 hours in air the ordinary filaments are almost completely separated from each other and the cilifers are clearly visible. There is no longer an obvious differentiation into descending and ascending tracts. Exposed at the upper right of the micrograph are the afferent and interconnecting vessels associated with the principal filaments.

Emersed gill (Plate 4.7). Breakdown of the gill structure is complete after 96 hours. In the lower left corner is the gill axis and disruption of the ordinary filament connections have caused the loss of any functional surfaces. At higher magnification (Plate 4.8) it is clear that most of the ciliated discs have separated and the ordinary filaments have disaggregated. This plate also

Plate 4.2

Pecten maximus. Ventral bend (VB) of unexposed gill. Bundles of densely ciliated ordinary filaments (OF), separated by supporting principal filaments (PF). Note the plicate, but structurally intact nature of the gill. Scale bar=100µm.

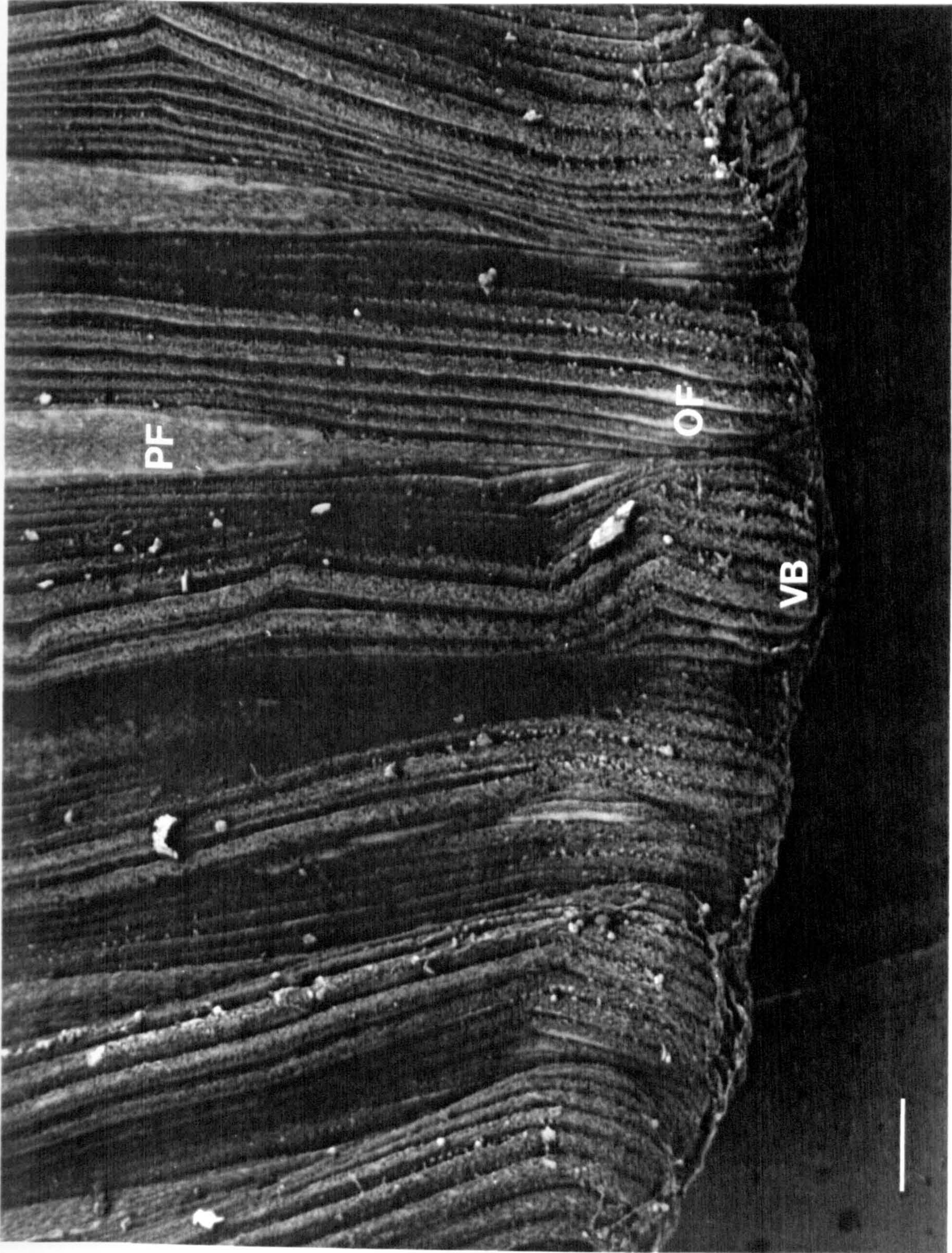


Plate 4.3

Pecten maximus. Ciliated spurs or cilifers on the abfrontal surface of unexposed gill. These structures maintain the structure of the gill by linking adjacent ordinary filaments with ciliated discs. Abbreviations as plate 4.2 and; c cilifer; cd ciliated disc. Scale bar=10 μ m.



Plate 4.4

Pecten maximus. Ventral bend and ascending tract of gill following emersion at 10°C and 95% r.h. for 24 hours. Abbreviations as plate 4.2 and; AV afferent vessel; IV interconnecting vessel. Scale bar=200µm.

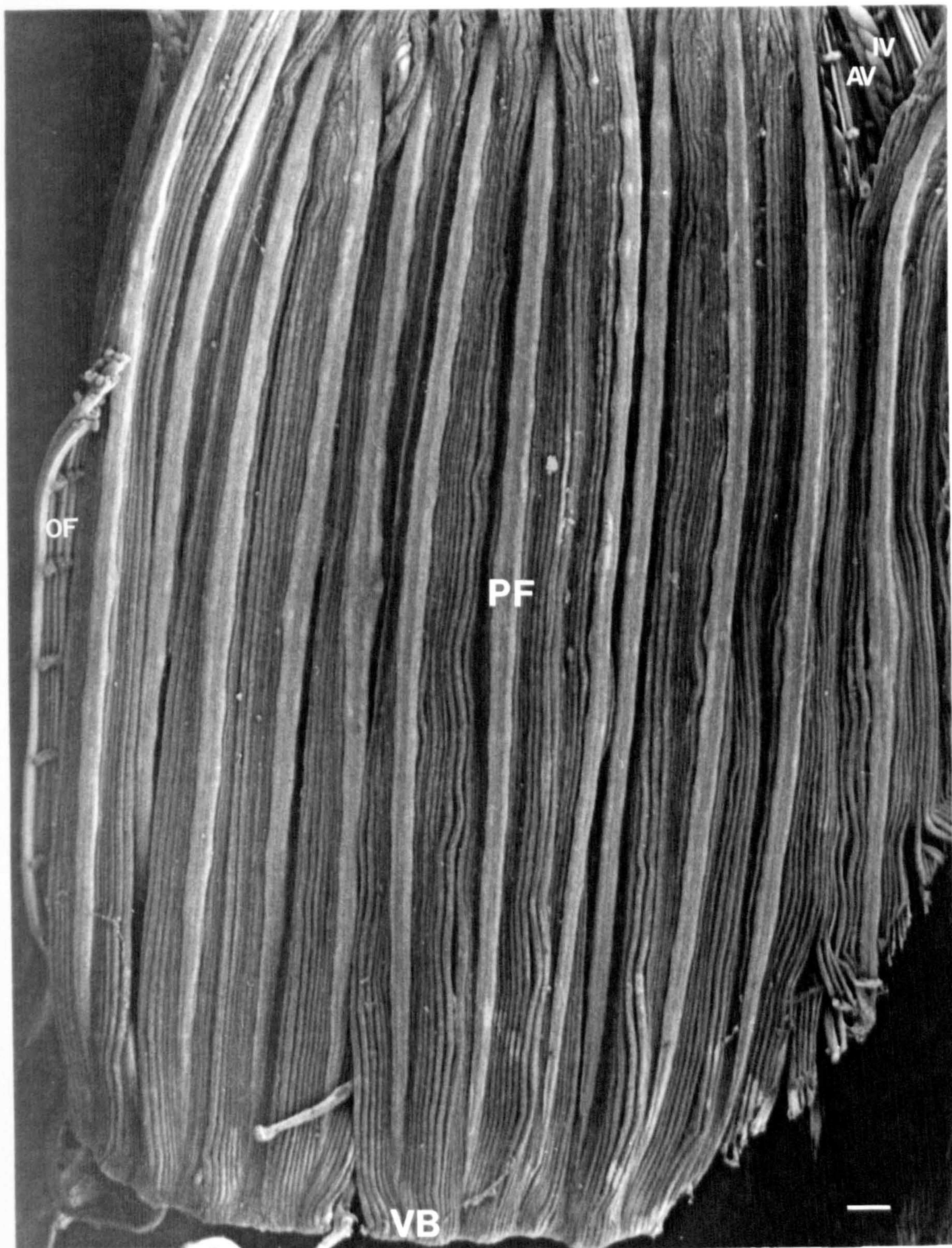


Plate 4.5

Pecten maximus. Ventral bend and lower ascending tract of gill following emersion at 10°C and 95% r.h. for 48 hours. Abbreviations as plates 4.2 & 4.3 and; m mucus. Scale bar=200µm.

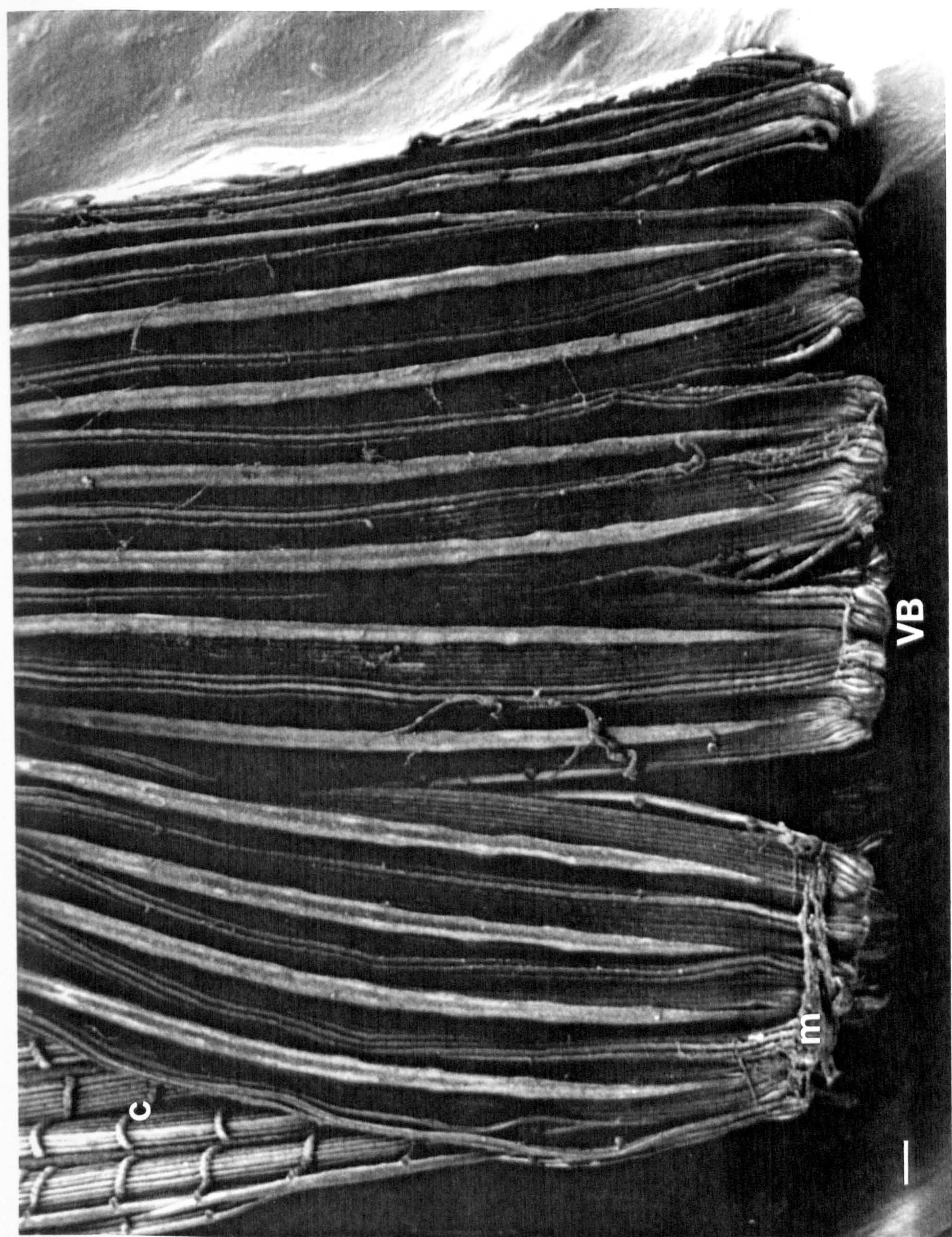


Plate 4.6

Pecten maximus. Frontal surface of gill following emersion at 10°C and 95% r.h. for 72 hours. Functional surfaces are no longer distinct. Abbreviations as plates 4.2-4.4. Scale bar=200µm.



Plate 4.7

Pecten maximus. Frontal surface of gill following emersion at 10°C and 95% r.h. for 96 hours. Gill structure completely broken down. Main reference point is the gill axis (ga) which has been cut approximately along the line of the branchial nerve (Fig. 4.10). Note rough texture of ordinary filaments due to the presence of numerous epithelial vesicles. Micrograph scale bar=100 μ m.

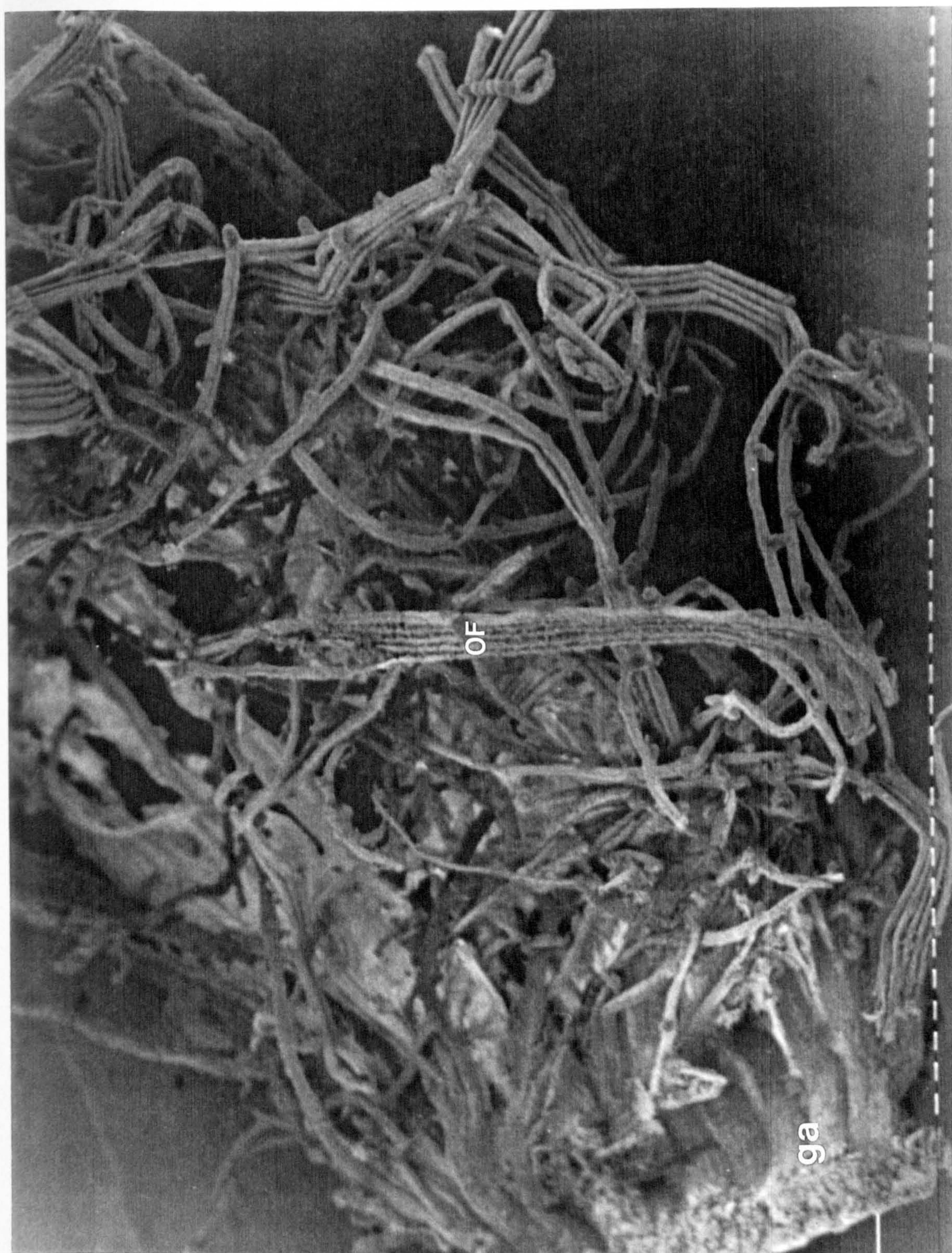
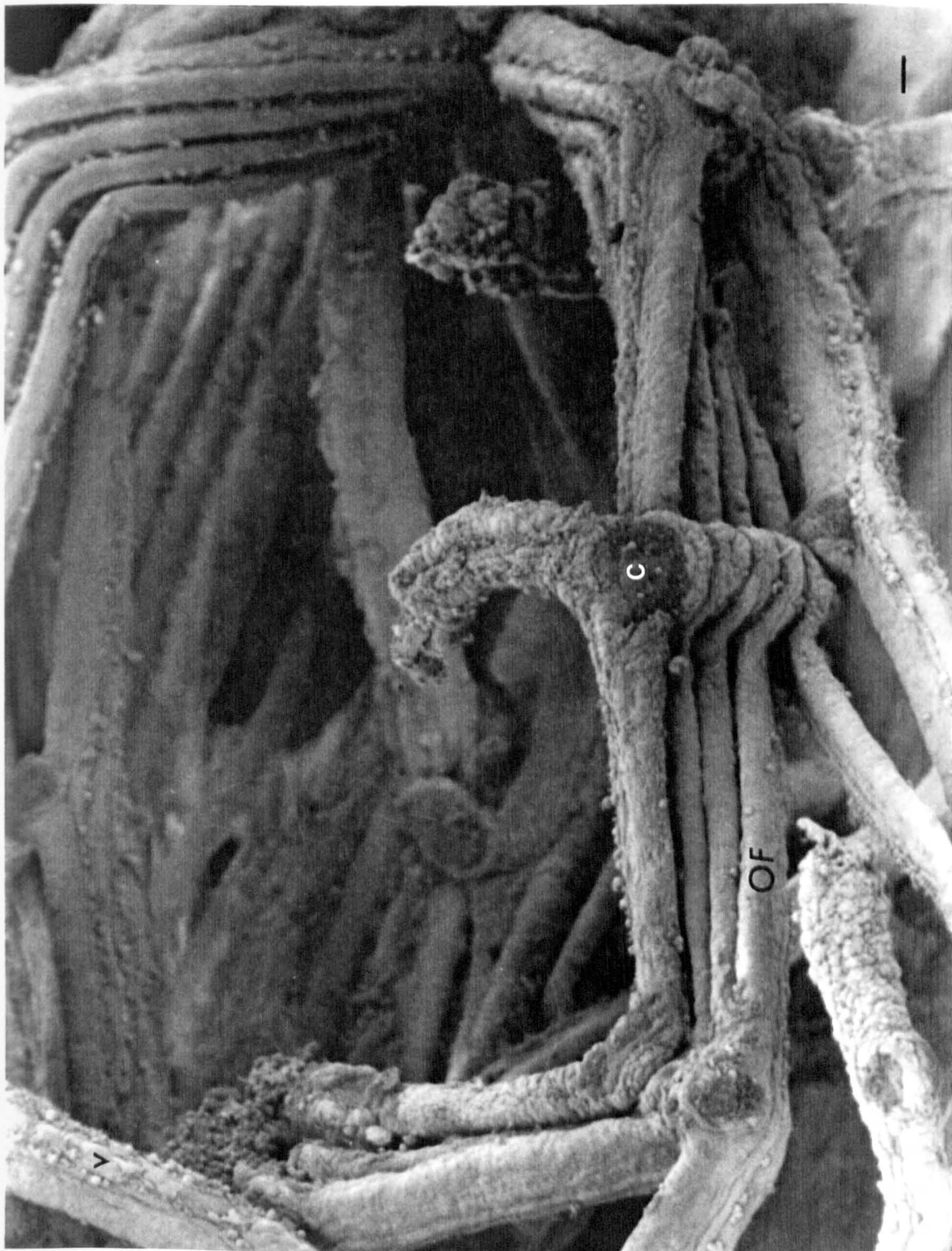


Plate 4.8 *Pecten maximus*. Ordinary filaments of the ascending tract of the gill following emersion at 10°C and 95% r.h. for 96 hours. Micrograph illustrates the extent of filament disaggregation and the presence of spherical vesicles (v). Abbreviations as plates 4.2-4.4. Scale bar=20µm.



illustrates another observation made of emerged scallop gills, the presence of numerous spherical particles or vesicles (v), ranging from 2 to 10 μm in diameter. These particles were not found on gills emerged for less than 48 hours and, although they appeared on all surfaces, they were most numerous on the proximal section of the descending tract, particularly the surfaces of branchial vessels (Plate 4.9). The particles were initially believed to be yeasts, or possibly bacteria, although the large size and uniformity of type made the latter hypothesis unlikely. Previous electron microscopy studies by Beninger *et al.*, (1988) and Le Pennec *et al.* (1988) identified spherical structures on the unexposed gills of *Placopecten magellanicus*. These particles, which were considered to be of two different types, one being mucus balls, the other thought to be secreted vesicles, may be similar to those observed in this study. If the particles were microorganisms, then they may be the cause, or an additional contribution, to the observed tissue breakdown during emersion. Transmission electron microscopy (T.E.M.) of sectioned gill tissue might indicate the presence of internal structures if the particles were microorganisms. Alternatively, if a T.E.M. study suggested an alternative explanation as to their nature, then a mechanism for ammonia toxicity may be provided.

4.10. Transmission electron microscopy of gill tissue

Materials and method

Since this work was primarily an investigation into the nature and potential physiological significance of the spherical structures observed during S.E.M. studies, progressive exposures were not carried out, and only control gills and gills emerged for 96 hours were examined. Specimen preparation for transmission electron microscopy (T.E.M.) was identical to S.E.M. preparation up to, and including, fixation with 0.5% uranyl acetate (section 4.9.). Thereafter, gill sections were rinsed with distilled water and dehydrated through a series of graded alcohols (ethanol) of 30, 50, 70% with 3 changes in each, during a 15 minute period. After 70% alcohol the tissue was transferred into a solution of 1% P-Phenylenediamine (PPD) in 70% alcohol and the dehydration procedure continued with 1% PPD in 90%, absolute and dried absolute alcohol. P-Phenylenediamine enhances the uptake of Osmium tetroxide which in turn improves the contrast of the specimen during microscopy. The tissue was then given three, 5 minute changes in 99% Propylene oxide (1,2-Epoxypropane), which replaced the absolute alcohol, before transferring to a 1:1 solution of Propylene oxide:Araldite epoxy resin (Araldite Cy212, 10ml; DDSA (hardener), 10ml; BDMA (adhesive), 0.4 ml, Agar Chemicals Ltd.). The lidless specimen vials were then placed in a rotometer, within a fume cupboard, which allowed the propylene oxide to evaporate overnight. Tissue samples were then transferred into moulds with fresh Araldite resin and oven dried at 60°C for 48 hours. The hardened tissue was then sectioned to a thickness of between 60-90nm on an Ultracut E microtome using a diamond

Plate 4.9

Pecten maximus. Outer surface of the branchial vessels following emersion at 10°C and 95% r.h. for 96 hours. Spherical particles appear in dense aggregations on both afferent (AV) and interconnecting vessels (IV). Micrograph scale bar=7μm.



knife. Finally, the tissue sections were stained in a 2% methanolic uranyl acetate solution (5 minutes), followed by a further 5 minutes in Reynold's lead citrate solution prior to observation and photography with a Zeiss 902 transmission electron microscope.

Results

Observation of transverse sections through the filaments and vessels of an unexposed gill revealed a number of cell types and a regular structure which could be related to the functional properties of the tissues. For example, Plate 4.10, a transmission electron micrograph of the frontal region of an ordinary filament, clearly illustrates the presence of 2 different epithelial cell types, the first having a relatively light, electron-clear cytoplasm and nucleus (CC), and the second being much darker with an electron dense cytoplasm and nucleus (DC). Also visible are numerous mucocytes enclosed in a cell membrane (M), although these cells may also appear free in the surrounding cytoplasm. The outer edge of this epithelium is bounded by microvilli (MV) which are formed from convolutions in the apical cell membrane. The microvilli have a continuous band of material covering their distal ends, possibly the cell coat or fuzzy coat, which appears to bridge the gap between microvilli. This coat, which contains a mucopolysaccharide component produced continuously by the epithelial cells, may function as a protective layer against bacterial invasion or sudden changes in the external environment (Toner & Carr, 1971). Frontal cilia (C) are also shown in this photograph, with the axial filament complex seen in transverse section. These cilia, which appear to originate from the electron-clear cells, are predominantly sensory and are sparsely distributed on the dorsal respiratory expansion (i.e. the area comprising the exposed branchial vessels, see Fig. 4.10). Cilia involved in the production of feeding and respiratory currents, which are a feature of the ascending tract and abfrontal surface of the descending tract, are much more densely aggregated and form regular, adjacent rows. The final feature of note in this plate is the presence of a number of small vesicles (V) around the bases of the microvilli.

All other sections of the different morphological structures of the gill were composed of these 3 cell types and contained microvilli and cilia to greater or lesser degrees depending on their particular function. Vesicles were not observed in all areas, although they were particularly common on the abfrontal region of the ordinary filaments.

Plate 4.11 shows a normal interconnecting vessel (c.f. plate 4.9) composed of a microvilli/ciliated outer cell layer, a number of dense cytoplasm nuclei, numerous cytoplasmic vesicles and golgi apparatus (G). The golgi apparatus is considered to be involved in packaging and secretion of various cellular products, in addition to polysaccharide synthesis, utilized in the cell coat of some microvilli.

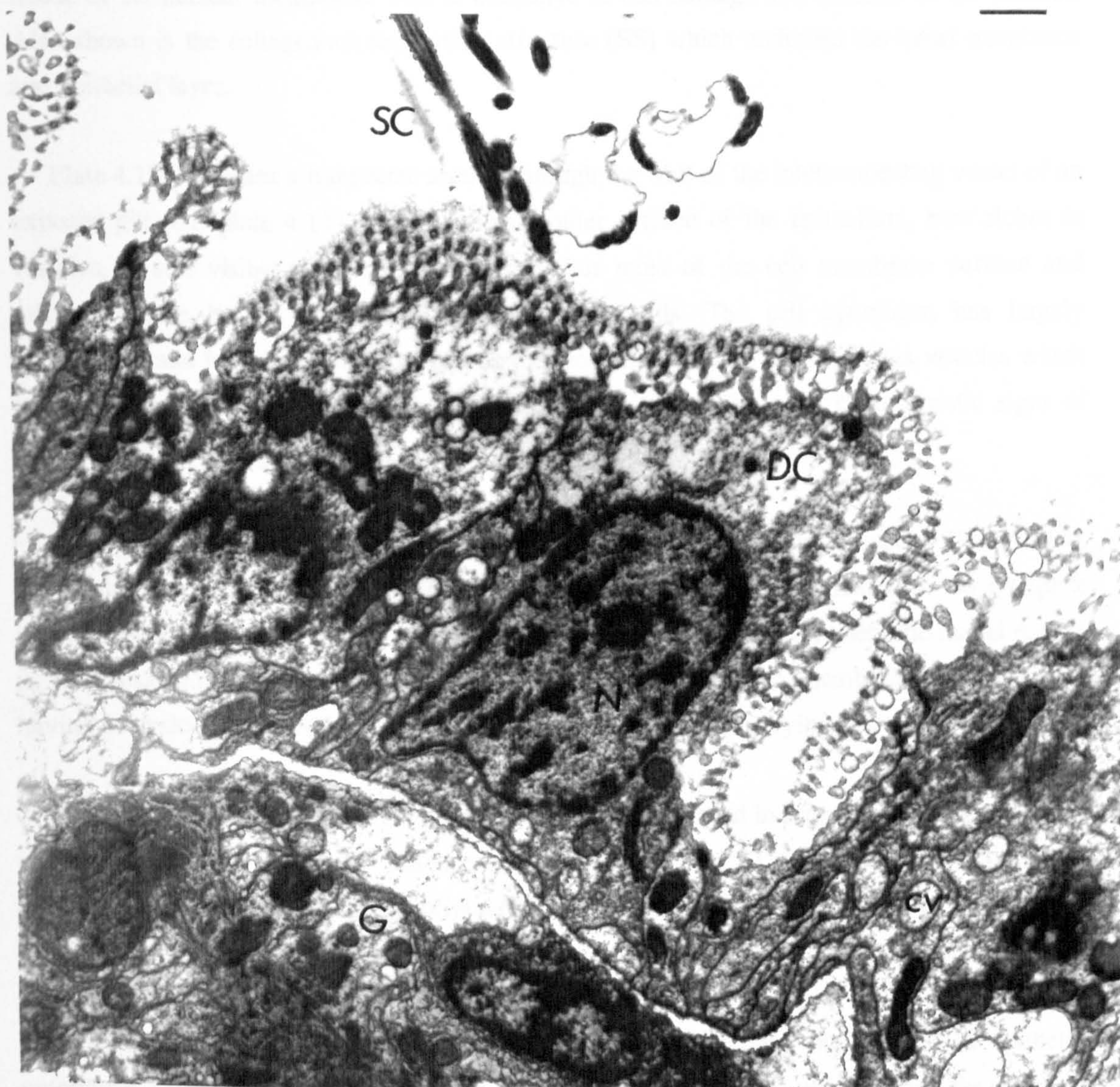
Plate 4.10

Pecten maximus. Section through the frontal region of an unexposed ordinary filament showing 2 epithelial cell types (CC&DC), membrane enclosed mucocytes (M), epithelial microvilli (MV), frontal cilia (C) and vesicles (v). Scale bar=1 μ m.



Plate 4.11

Pecten maximus. Section through unexposed interconnecting vessel. Micrograph shows electron dense epithelial cell type (DC) with nucleus (N). The convoluted outer surface is covered with microvilli and occasional sensory cilia (SC). Numerous cytoplasmic vesicles (cv) and golgi apparatus are visible (G). Scale bar=1 μ m.



Examination of gill tissue emersed for 96 hours revealed that a substantial alteration in the epithelial cell structure had taken place (Plate 4.12). This micrograph, of a similar area of ordinary filament to plate 4.10, shows that, while the cell nuclei are still visible, little of the cytoplasm remains and the microvilli and associated cell membrane are no longer present. In addition, the vesicles have increased in size and number and they appear both inside the remnants of the cell and on the outer surface. The nuclei themselves are also enlarged and the nuclear material is now visible as concentrated areas of electron dense material around the inside of the nuclear membrane. This is indicative of cell damage and necrosis of the nucleus. Also shown is the collagenous supporting structure (SS) which underlies the basal membrane and epithelial layer.

Plate 4.13 illustrates a transverse section through the wall of the interconnecting vessel of an exposed gill (c.f. plate 4.11). The convoluted outer surface of the epithelium, now richer in vesicles, is still visible on part of the vessel, but most of the cell membrane surface and microvilli have broken away from the underlying cells. The cell cytoplasm has largely disappeared and is now replaced by, or possibly contained within, the numerous vesicles which extend down to the basal membrane itself. Cell nuclei again show the characteristic signs of necrosis, that is, enlarged size and marginal deposition of nuclear material.

At higher magnification the extent of vesicle concentration can be observed (Plate 4.14). This micrograph, showing a section through an ordinary filament, illustrates the complete breakdown of the gill epithelial layer following a four day emersion. Only necrotic nuclei remain as recognisable cellular components, the remainder of the cytoplasm, membranes and microvilli having been sloughed off or possibly enclosed to form vesicles of varying size.

T.E.M. studies indicated that the spherical structures observed in scanning micrographs were not microorganisms, due to the absence of organelles or internal structure. Plate 4.14 shows vesicles within the size range of the original structures, but without the presence of recognisable mucocytes it seems unlikely that they are equivalent to the mucus balls referred to earlier. However, the massive cellular deterioration observed in exposed gill tends to support the alternative explanation that these structures are vesicles containing the remains of epithelial cytoplasm and organelle material. Further support for this view comes from the work of Nuwayhid (1978) who reported superficially identical structures, both in S.E.M. and T.E.M. studies, on the gills of *Patella vulgata* following exposure to oil dispersant compounds. The similarity between this work and the present study, particularly micrographs such as Plate 4.15, which illustrates a cytoplasmic vesicle being extruded from the remnants of the epithelial layer, is striking. It would appear from this evidence that necrosis of gill epithelial-cells, whether caused by chemical pollutants, metabolic waste products, such as ammonia, or bacterial activity, has a similar pattern of breakdown.

Plate 4.12

Pecten maximus. Section through the frontal region of an ordinary filament following emersion at 10°C and 95% r.h. for 96 hours. Nuclei are enlarged with marginal deposition of nuclear material. Numerous vesicles extend from the collagenous supporting structure (SS) to the remnants of the microvilli layer. Scale bar=1 μ m.

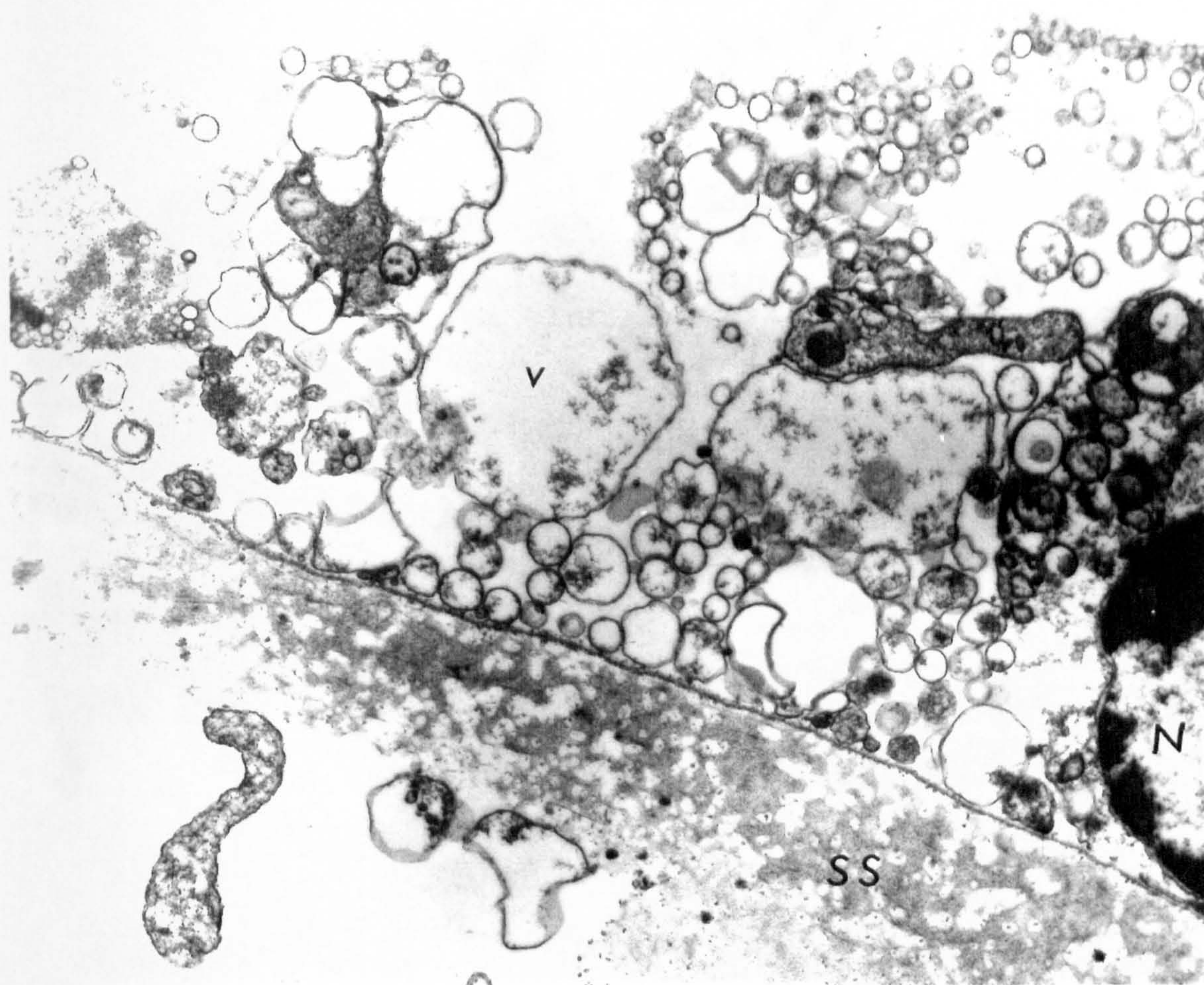


Plate 4.13

Pecten maximus. Section through an interconnecting vessel following emersion at 10°C and 95% r.h. for 96 hours. Necrotic nuclei show marginal deposition of nuclear material and most of the epithelial layer has been lost exposing cytoplasmic vesicles and basal supporting structure. Vesicles are also concentrated in the microvilli layer. Abbreviations as plates 4.10-4.12. Scale bar = 2µm.

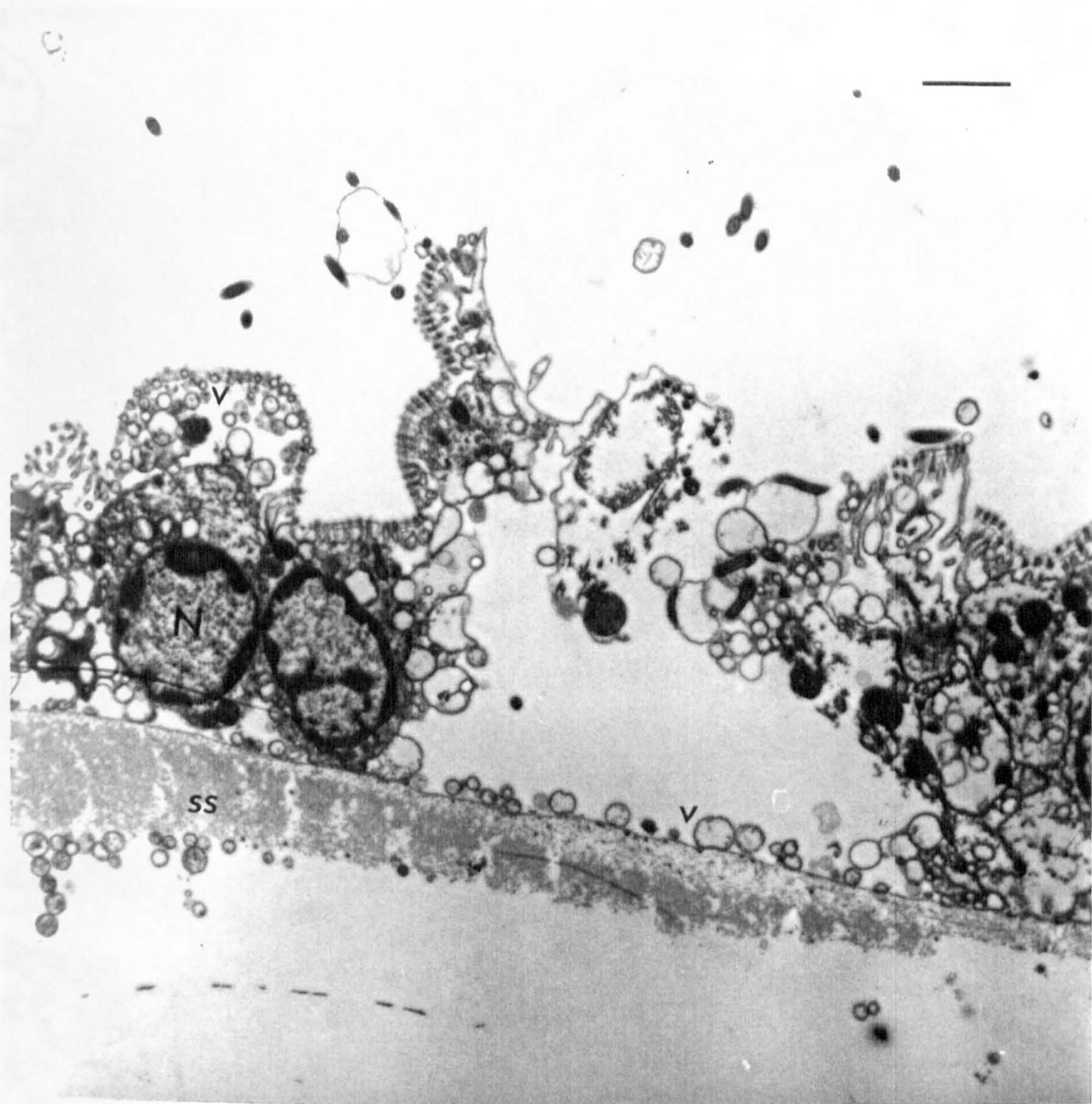


Plate 4.14

Pecten maximus. Section through an ordinary filament following emersion at 10°C and 95% r.h. for 96 hours. At high magnification only necrotic nuclei are recognisable. The cytoplasmic space between the remnants of the micovilli layer and the collagen supporting layer is composed almost entirely of vesicles. Abbreviations as plates 4.10-4.12. Scale bar = 1µm.

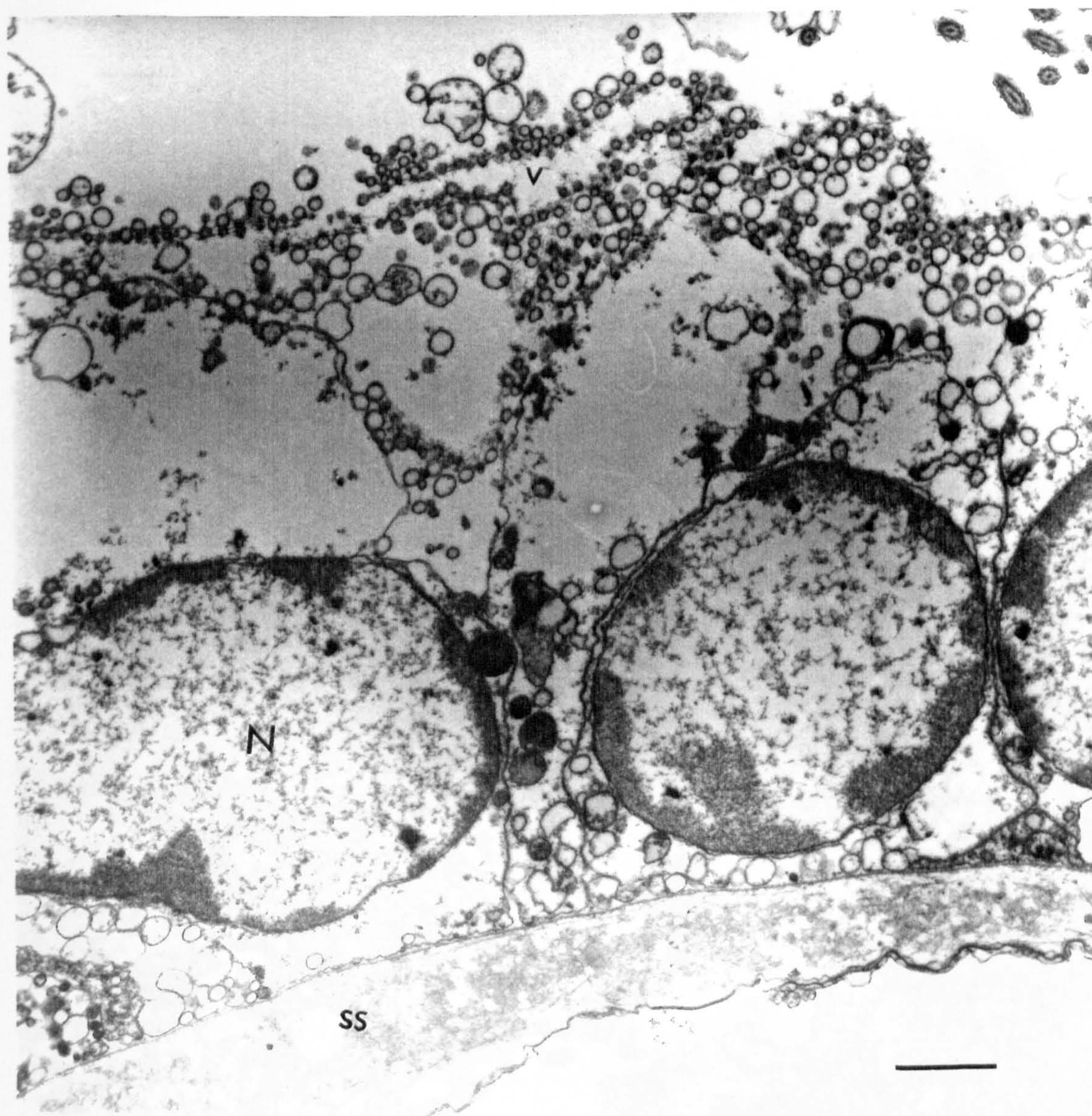


Plate 4.15

Pecten maximus. Section through an ordinary filament following emersion at 10°C and 95% r.h. for 96 hours. Large cytoplasmic vesicle (cv) being extruded from the epithelial layer. Smaller vesicles are distributed throughout the remaining cell cytoplasm and particularly around the microvilli layer. Abbreviations as plates 4.10-4.12. Scale bar = 1 μ m.



Discussion

The scallop *Pecten maximus* exhibits the common relationship amongst bivalves of a variation in heart rate in response to acute changes in environmental temperature. This response is shared with many other species including; *Mytilus edulis* (Widdows, 1973), *Isognomon alatus* (Trueman & Lowe, 1971), *Mya arenaria* (Lowe & Trueman, 1972) and *Modiolus modiolus* (Coleman, 1976) and has been interpreted as being indicative of changes in overall metabolism (Bricelj & Shumway, 1991).

It is interesting to note, therefore, that in *Pecten maximus*, heart rate was found to show a linear increase only between 4 and 19°C, and at higher temperatures the relationship broke down and became irregular. The Q_{10} value, a widely used measure of the relationship between physiological rates and temperature, also indicated that heart rate became less temperature dependant towards the upper limits of the experimental range. Similar results have been reported by Lowe & Trueman (1972) and Coleman (1976) for *Mya arenaria* and *Modiolus modiolus*, and it appears to reflect an inability in these species to maintain a heart rate compatible with higher rates of metabolism. If the heart is forced to work maximally for extended periods then it seems likely that eventual failure will result. The upper limit of the range of cardiac function in *Pecten maximus* coincides broadly with the upper lethal temperatures, between 19 and 24°C, determined for other sub-littoral, temperate bivalves (Dickie, 1958; Read & Cumming, 1967; Paul, 1980). If this response is related to the survival ability of *Pecten maximus* during emersion at different temperatures, and assuming that metabolic responses to temperature are similar in air and water, then these data tend to support the earlier hypothesis that poor survival at higher temperatures is related to two main factors. Firstly, as environmental temperature approaches a certain level, coordination of physiological systems, such as cardiac function, will deteriorate, partly due to limitations of the respiratory system to provide sufficient oxygen to maintain energy production, and hence muscular function, and partly due to the functional capacity of the tissues themselves. Secondly, a higher metabolism, caused by high environmental temperature, results in increased production of end products which cannot be easily disposed of during emersion and consequently they accumulate to harmful levels. Beyond this temperature, corresponding approximately to the upper lethal temperature, metabolite accumulation may become acutely toxic and physiological systems are unable to respond to further demands, resulting in complete failure.

The actual metabolic responses to increasing temperature during emersion are rather more difficult to isolate, since indicative metabolic parameters, such as heart rate, are likely to be affected by adduction activity and the loss of hydrostatic support for the viscera and heart. Brand & Roberts (1973) implicated loss of support as a cause of heart arrhythmia in scallops during emersion. Therefore, during emersion, the separation of such complicating factors from a

simple heart rate/temperature relationship may not be possible, and the assumption that variation in air temperature will have the same effect on metabolism as changes in water temperature, can only be tested in the absence of these additional complicating factors. Therefore, although based upon the physiological response to changes in aquatic temperature, this model appears to fit the observed situation during emersion equally well. Higher air temperatures will tend to increase metabolism, and therefore the difficulty of meeting greater metabolic demands from poorly adapted respiratory and circulatory systems will only be compounded by additional activity, such as adduction, and any reduction in cardiac efficiency as a result of the loss of hydrostatic support. It is therefore important, during harvest and subsequent transit, that wide temperature fluctuations and high exposure temperatures are avoided and that storage, close to the previously determined optimum, is maintained.

The cardiac responses of *Pecten maximus* during emersion under constant conditions are also important, since, although limited as a simple indicator of metabolic rate, the heart must still act to maintain haemolymph and oxygen distribution via the circulatory system. Any deterioration or reduction in this activity may have implications for survival during emersion. Although individual heart-rate responses during emersion were variable, the most common pattern observed was that of a brief tachycardia immediately upon exposure, followed by a relatively rapid bradycardia to a rate which was maintained, or declined very gradually, throughout the remainder of the exposure period. This general response is similar to that reported by Coleman & Trueman (1971) for the predominantly sub-littoral mussel, *Modiolus modiolus*, and by Brand & Roberts (1973) for the present species. In the latter study, the brief increase in heart rate was associated with the initial burst of adduction activity forcing a greater haemolymph volume into the heart, which in turn caused stretch receptors in the ventricle wall to increase heart rate (Hill & Welsh, 1966). The higher cardiac output, as a result of tachycardia, will also be required for delivery of additional oxygen to supply the increased activity of the adductor muscles. Thompson *et al.*, (1980) also associated the heart rate increase, observed during valve adduction in the scallop *Placopecten magellanicus*, with this function. In addition, they concluded, from the evidence of low blood PO_2 and the subsequent resort to anaerobic pathways, that this increased heart rate, which was maintained throughout the recovery period, was still insufficient to meet the energy demands of the animal even in fully aerated sea water. When the present situation is considered, of an emersed scallop exhibiting a similar activity pattern, it seems even more unlikely that energy demands could be met by aerobic metabolism, particularly since both heart rate and haemolymph PO_2 decrease during the emersion period. These two parameters may actually act antagonistically since environmental PO_2 has been substantially linked with changes in the heart rate of a number of marine bivalves (Booth & Magnum, 1978). For example, Brand & Roberts (1973) showed that under conditions of slowly declining oxygen tension in water, *Pecten maximus* maintained heart rate down to 25mm Hg (16% saturation). Below this value heart activity declined rapidly. However, under

conditions of rapidly-induced hypoxia or emersion, the pattern of heart activity was similar to this study, in that a bradycardia was recorded immediately following the initial rate increase.

The causes of the reduced heart rate during these two conditions were, however, considered to be different since only during rapidly-induced hypoxia would the scallop quickly reach the critical point or P_c of 25 mm Hg (Torr), i.e the point at which maintenance of heart rate deteriorated. They proposed instead, that bradycardia during emersion was related more to mechanical stress of the heart as water was lost from the mantle cavity during adduction. This assumes, however, that the aquatic P_c for heart rate is applicable during emersion and that the cardiac response to a rapid reduction in haemolymph PO_2 is the same as to a gradual decrease. Additionally, bradycardia during emersion is widespread in bivalves, the few exceptions being those species which have an air-breathing capacity sufficient to maintain internal PO_2 close to, or equal to, normoxic conditions. Some of these species, for example *Cardium edule*, expel most of their mantle-cavity water to enable air breathing during emersion (Boyden, 1972b). Since this behaviour is similar to that shown by *Pecten maximus* it might be expected that a similar loss of cardiac support would result in a heart-rate decrease. The fact that it does not implies that haemolymph PO_2 is important in the initial bradycardia and that the decrease in heart rate shown by *Pecten maximus* relates partly to the rapid decline in oxygen uptake which is evident from the onset of emersion.

Based on heart activity, therefore, emersion in *Pecten maximus*, seems to correspond better to a rapid hypoxia rather than a gradual fall in oxygen tension, implying that the respiratory system is compromised from the onset of emersion. Consequently, any further deterioration of respiratory tissues by external factors, which result in a lower internal PO_2 , may act to reduce heart rate further, thus compounding the situation and eventually leading to complete cardiac arrest.

As mentioned briefly above, bivalves adapted for life in the littoral zone exhibit rather different cardiac responses to emersion, reflecting adaptation to their relative shore position. For example, the high-littoral oyster *Isognomon alatus* (Trueman & Lowe, 1971) and *Polymesoda caroliniana* (Deaton, 1991) show no heart rate reduction in response to emersion due to their ability to absorb atmospheric oxygen across moist tissues, exposed by controlled gaping of their shell valves. In the latter species, the rate of oxygen uptake in air is identical to that during immersion and presumably haemolymph PO_2 is also little changed. As a result, fully aerobic metabolism is possible during normal activity levels without recourse to anaerobic pathways. Further down the shore, the mid-littoral species, *Mytilus edulis*, whose air breathing ability is less well developed, shows a marked, and relatively rapid bradycardia, which is related to a decrease in mantle cavity PO_2 as the animal closes its shell valves for the duration of the exposure (Helm & Trueman, 1967; Coleman & Trueman, 1971). The mussel then undergoes a

period of anaerobiosis (Shick & Widdows, 1981) and conserves energy by maintaining a low heart rate which is no longer required to circulate oxygenated haemolymph. Upon reimmersion, a higher blood PO_2 is quickly established and a rapid tachycardia overshoots the normal heart rate for the purposes of re-metabolising the accumulated anaerobic end products.

In contrast, Coleman & Trueman (1971) also investigated the primarily sub-littoral mussel *Modiolus modiolus*, which showed a similar cardiac response to emersion as *Pecten maximus*. In this case, however, a much more gradual bradycardia, accompanied by periodic adductions, eventually resulted in a low and irregular heart rate which was slow to recover when the animal was reimmersed. Also in common with *Pecten maximus*, the adduction activity of this species, and an inability to control shell gape or seal its shell valves, resulted in the loss of mantle cavity water during emersion. When compared with the littoral species mentioned previously, which generally retain mantle water, either via controlled gaping or actively sealing the water within the shell, the importance of water retention as a littoral adaptation becomes clear. Not only does the bivalve avoid the effects of desiccation of respiratory and circulatory organs, but it may also gain hydrostatic support which enables the animal to prevent physical damage of the gills and heart, enabling rapid recovery when reimmersion occurs. Although *Mytilus edulis* reduced heart rate, since the capacity to circulate dissolved oxygen was no longer required, the organ was still maintained in a supportive medium for the duration of the exposure.

Additionally, sub-littoral species tend to show increased activity, due to adduction, and consequently do not actively reduce heart rate during emersion. Without the support of water, which is expelled during valve adduction, the gills collapse and oxygen uptake decreases resulting in a reduction of haemolymph PO_2 which in turn causes heart rate to fall. The observed reduction in heart rate is likely to be compounded by direct damage to the heart and the higher energetic cost of haemolymph circulation through a compressed vascular system as hydrostatic support of the body is lost. This hypothesis is partially supported by measurements of oxygen uptake and haemolymph oxygen concentration during emersion, which indicate rapid initial reductions as the gill filaments clump together, followed by gradual declines as heart function slowly deteriorates.

The aquatic respiration rate of *Pecten maximus* was similar to those obtained by Brand & Roberts (1973) ($150\text{--}250 \mu\text{l g}^{-1} \text{h}^{-1}$) for the same species, and also falls within the range of measurements obtained for other comparable pectinid species (Spärck, 1936; Vahl, 1972; 1978; Mclusky, 1973; MacDonald & Thompson, 1986). Rates of oxygen uptake for emersed pectinids again appear to be lacking, perhaps not surprisingly since members of this family are predominantly sublittoral. However, comparison with species better adapted to survive aerial exposure is useful, and may be relevant in determining likely causes of death, particularly when linked to other physiological parameters. The respiration rates of emersed *Pecten maximus*

showed a gradual decrease to about 28% of the rate recorded in water, which is slightly lower than the ratio of 0.37:1, i.e. 37%, determined for the aerial:aquatic $\dot{V}O_2$ in sub-littoral molluscs generally (McMahon, 1988). By comparison, this same study, and others (notably Deaton, 1991), have shown respiratory ratios for high-littoral bivalves ranging from 0.6 to 0.8:1, and exceptionally as high as 1:1. These higher ratios, and the very low rates of anaerobic end product accumulation (Widdows *et al.*, 1979; Nicchita & Ellington, 1983; Deaton, 1991) indicate largely aerobic metabolism, except for a partial reliance on anaerobic pathways in the deep tissues of the adductor muscle during activity (Booth & Magnum, 1978). Much lower aerial respiration rates are recorded in mid and low-shore bivalves, largely due to the behaviour of actively sealing their shell valves during emersion, thus preventing access to oxygen. Consequently these bivalves resort almost exclusively to anaerobic pathways. This results in respiratory ratios below 0.15:1 for species such as *Mytilus galloprovincialis* (Widdows *et al.*, 1979) *Cerastoderma glaucum* (Boyden, 1972b) and *Choromytilus meridionalis* (Griffiths, 1981), and as low as 0.05:1 for *Mytilus edulis* (Widdows *et al.*, 1979). If the respiratory and cardiac data of these species are combined, then the typical pattern of physiological response to emersion in mid and low-littoral bivalves is observed. Shell valves are generally closed, or the gape much reduced, thus preventing oxygen uptake from the air and resulting in a severe reduction of $\dot{V}O_2$. As a consequence, the oxygen concentration of the retained mantle cavity fluid falls to only about 10% of saturation, causing a reduced heart rate which may become progressively slower depending on the duration of the emersion (Helm & Trueman, 1967). A reduction in activity and metabolism enables anaerobic pathways (Widdows *et al.*, 1979; Klutymans *et al.*, 1977, 1978) to supply the energy requirements of the animal during both short tidal emersions and extended exposures (Wijsman, 1976). The lower metabolic rate and tolerance of anaerobic end-product accumulation allows conservation of energy reserves and confers an ability to survive for long periods in air.

The data from the present study suggest that exposure brings about similar physiological changes in sub-littoral bivalves, but that different behavioural and metabolic responses at least partially explain the relatively poorer survival of these species. *Pecten maximus* does not seal its shell valves during emersion, but instead undergoes a period of rapid valve adduction, which in other species has been associated with mantle cavity ventilation (McMahon, 1988; Byrne *et al.*, 1990). Despite access to atmospheric oxygen, and behaviour that actively ventilates the mantle cavity, the respiration rate decreases suggesting problems of gas uptake rather than reduced oxygen availability in the mantle cavity. It seems likely that this is related to a large decrease in the gas exchange surface area as the gill filaments collapse and clump together following expulsion of the fluid from the mantle cavity space.

Additionally, if water expulsion does reduce cardiac support, then $\dot{V}O_2$ may reduce, due to inefficient heart action causing a decrease in the circulation of haemolymph oxygen.

Consequently, internal PO_2 would decrease, perhaps further suppressing heart rate (Bayne, 1971; Brand & Roberts, 1973) and again contributing to the overall reduction in $\dot{V}\text{O}_2$. Following the initial rapid decreases in $\dot{V}\text{O}_2$ and haemolymph PO_2 shown in this study, these parameters continued to decline, although more gradually, indicating a long term deterioration in respiratory and circulatory systems. This gradual decrease in the diffusion of oxygen through surface tissues may result from epithelial cell death, caused by the accumulation of toxic compounds produced by the scallop and surface bacteria. It was observed during haemolymph sampling that very few of the scallops were able to maintain cardiac function beyond 72 hours, and it seems that lack of oxygen, combined with loss of hydrostatic support and the effort to maintain blood flow to active tissues, are the likely causes of heart failure.

Partial control of bacterial growth by the use of antibiotics indicated that $\dot{V}\text{O}_2$ declined further than the initial work had suggested (Fig. 4.4). In comparison with antibiotic treated animals, scallops emersed without treatment have a bacterial respiratory component sufficient to cause a significant increase in the respiration rate after only 3 days. The build up of a large bacterial population within the mantle cavity and on tissue surfaces may well make an important contribution to the gradual long-term decline in $\dot{V}\text{O}_2$ by damaging delicate gill and tissue epithelia. The results of an investigation into the effects of bacteria on such tissues will be detailed in the following chapter.

An additional factor which may also be relevant in the context of tissue damage and resultant physiological disruption, is that of the scallop's own excretory products. Gas exchange surfaces must operate in both directions in order to remove the products of aerobic metabolism, i.e. CO_2 , otherwise respiratory acidosis will occur. Measurement of haemolymph pH indicated that an acidosis developed during emersion, a feature also recorded in other bivalves (de Zwann & Wijsman, 1976; Byrne *et al.*, 1991; Byrne & McMahon, 1991). In other species, notably *Mytilus edulis*, acidosis may have a substantial metabolic component as a result of organic acid production during anaerobic metabolism (de Zwann *et al.*, 1983). However, a more detailed study of emersion induced acidosis in this *Pecten* species (Duncan *et al.*, in prep.) found little evidence of organic acid accumulation in the haemolymph, a situation similar to that reported for *Placopecten magellanicus* (Thompson *et al.*, 1980). This absence of significant quantities of organic acids suggests that little anaerobic metabolism occurs in superficial tissue, although Booth & Magnum (1978) have suggested that deep, active tissue, such as adductor muscles, are predominantly anaerobic during exercise. If this is the case, then the results of our studies suggest, in addition to the conclusion of de Zwann *et al.*, (1980) that there is little transport of biochemical compounds within the muscle, that there is also minimal translocation from muscle to haemolymph. Therefore, the observed haemolymph acidosis seems exclusively respiratory in origin and confirms the earlier hypothesis that CO_2 exchange at the respiratory surfaces is compromised. The pH decrease in the haemolymph was consistent throughout the emersion

period suggesting that the internal buffering capacity of the animal was limited. Many bivalve species are capable of mobilising shell carbonates during emersion in order to limit the extent of acidosis (Crenshaw & Neff, 1969, Akberali *et al.*, 1977; Byrne & McMahon, 1991), however, this ability appeared to be absent in *Pecten maximus* and was later confirmed experimentally (Duncan *et al.* in prep.). Thompson *et al.* (1980) also reported the absence of shell buffering in the scallop *Placopecten magellanicus* during a respiratory acidosis induced by escape swimming. The consequences of an uncontrolled acidosis are serious, since even the smallest alterations in H^+ concentration can have a great influence on the rates of enzyme activity and the structure of protein molecules, principally through ionization effects which prevent normal physiological function (Hochachka & Somero, 1984).

If anaerobic pathways are utilised in the muscles, which seems probable given that oxygen consumption decreased during a period of high activity, then what are the consequences of end-product accumulation within the muscle tissue, particularly since little translocation seems to occur and there is no opportunity for aerobic recovery? It is likely that continued muscular activity would be difficult, due to the problems of cellular pH and osmotic imbalance, and the limited ability of sub-littoral bivalves to sustain anaerobic energy production over an extended period. Any resultant muscle failure will cause uncontrolled gaping of the shell valves and, as a significant quality feature of the live scallop market, warrants further investigation.

The final source of potentially damaging end products will come from nitrogen metabolism, i.e. the catabolism of amino acids. The principal nitrogenous end-product of marine bivalves is ammonia (Potts, 1967) which is both highly toxic and highly soluble. Ammonia has been found to accumulate in the mantle-cavity fluid and haemolymph of a number of bivalves during emersion (Akberali, 1977; Bayne *et al.*, 1976; Thompson *et al.*, 1978; de Zwann *et al.*, 1983) but, in common with other metabolic parameters, the rates of excretion and accumulation vary depending on the degree of littoral adaptation and the behavioural responses exhibited. For example, Widdows & Shick (1985) compared the two intertidal species *Mytilus edulis* and *Cardium edule*, which differed principally in their behaviour during aerial exposure. As discussed earlier, *M. edulis* tends to close its shell valves and reduce metabolism in air, and this is reflected in ammonia excretion rates. In contrast, the valves of *C. edule* gaped during emersion, which enabled oxygen uptake to be maintained at 50-75% of the aquatic rate by air breathing, and ammonia excretion rates were equal or even slightly higher than those of constantly immersed individuals. Valve gaping bivalves in general, appear to maintain higher rates of ammonia excretion during exposure (Jordan & Valiela, 1982), presumably as a consequence of their relatively higher metabolic rate sustained, to varying degrees, by aerobic pathways. This assumption is supported by the work of Thompson *et al.* (1980) which showed that higher activity, and hence metabolism, resulted in increased ammonia production in the scallop *Placopecten magellanicus*. Therefore, *Pecten maximus*, as an active, valve gaping

bivalve might be expected to accumulate a relatively high ammonia concentration during emersion.

Additionally, Widdows & Shick (1985) reported that individuals of *M. edulis* and *C. edule*, exposed to different littoral conditions prior to emersion, showed significantly higher than normal rates of metabolism and ammonia excretion when acclimated to subtidal conditions, but lower rates if previously adapted to an intertidal regime. This illustrates the importance of littoral adaptation in the control of excretory product accumulation during emersion and implies that *Pecten maximus*, with no experience of exposure, will again build up relatively higher concentrations of ammonia over a shorter time. Both hypotheses appear to be confirmed when the results of this study are compared with those obtained for the mussel *Mytilus californianus* (Bayne *et al.*, 1976, Thompson *et al.*, 1978). The subtidal scallop, in contrast to the mussel, maintains a relatively constant rate of ammonia excretion, and hence nitrogen metabolism, throughout the emersion period. The rate of production is also considerably higher than that of *M. californianus*, reaching a mantle fluid concentration of $91.2 \mu\text{mol.l}^{-1}$ after 6 hours emersion, compared with approximately $1.7 \mu\text{mol.l}^{-1}$ in the mussel. Ammonia accumulation in the blood is also markedly greater in *P. maximus*, reaching $320 \mu\text{mol.l}^{-1}$ after 35 hours in air, but only $29.4 \mu\text{mol.l}^{-1}$ in *M. californianus*. An increased blood ammonia concentration has also been reported in another scallop species, *Placopecten magellanicus* (Thompson *et al.*, 1980) and was associated with a period of rapid valve adduction. Although these particular experiments were carried out in water, it seems likely that such activity is also responsible for the observed accumulation in *P. maximus*, and lends additional support to the hypothesis that during emersion this species maintains, or attempts to maintain, an active metabolism.

In bivalves which normally experience conditions of tidal exposure any accumulated ammonia is rapidly excreted when the animal is reimmersed, and reaches pre-exposure levels in the mantle cavity fluid within a few minutes (Bayne *et al.*, 1976), and in the blood within a few hours (Thompson *et al.*, 1978; Widdows & Shick, 1985). However, this recourse is not available to the scallop in transit, with the consequence that ammonia concentrations will continue to increase throughout the emersion period, reaching potentially lethal levels. Ammonia is known to be toxic to almost every form of life, from plants to vertebrates, but its toxicity is generally considered to be dependant on the chemical state and the physical condition of the medium that the organism is exposed to. Ammonia exists in two forms, un-ionised (NH_3) and ionised (NH_4^+), with the un-ionised form the more toxic (Campbell, 1991). The relative proportions of these forms in solution are determined by the pH (Warren, 1962), and, to a lesser extent, the temperature (Trussell, 1972), with lower pH and temperature producing more NH_4^+ and hence being less toxic (Spotte, 1970). The situation is complicated still further since it appears that at any given pH, low oxygen concentrations enhance the toxicity of ammonia, both in fish (Merkens & Downing, 1957) and mammals (Warren & Schencker, 1960), possibly by

action on the nervous system. Although this hypoxia effect has not yet been investigated in other animal groups it is possible that invertebrate species exhibit a similar response and it may therefore be of relevance to emerged scallops.

Given that physico-chemical parameters can alter the toxicity of ammonia, even the presence of relatively low concentrations of NH_3 can have acute and long term detrimental effects on aquatic organisms. For example, Merkens & Downing (1957), Becker & Thatcher (1973) and Chin & Chen (1987) reported acute toxicity effects for NH_3 between 1 and 10 parts per million (ppm), while sub-lethal effects may be apparent in concentrations as low as 0.007ppm NH_3 (Burrows, 1964). Studies relating specifically to ammonia toxicity in bivalves are limited, although Epifanio & Srna (1975) investigated the effects of this compound on the clam, *Mercenaria mercenaria*, and the oyster, *Crassostrea virginica*. The results of these experiments indicated that, for these species, the 96 hour median tolerance limits (96h TLm) were 1.1×10^{-2} M and 8.2×10^{-2} M (110 & 880 ppm) respectively, concentrations which are remarkably high. However, the experimental protocol followed in the study does not indicate whether or not the desired ammonia concentrations were maintained throughout the 96 hour period, stating only that the initial exposure concentrations were measured. Since ammonia is broken down relatively easily by both chemical and bacterial action, and tends to escape from solution, it would be necessary to continuously supply experimental tanks with the required concentrations in order to ensure a constant experimental condition, otherwise ammonia concentrations would gradually decline and the organisms would appear to have a much higher tolerance to the compound. The procedure adopted in this present study ensured a relatively constant level of exposure and the 96h TLm for *Pecten maximus* was determined as 1.86 mM total ammonia (31.60-33.46 ppm, dependant on whether NH_4^+ or NH_3 is used in the calculation), of which 2.01-3.0% constituted NH_3 . The percentage of un-ionised ammonia (% UIA) in this study, which was determined according to Whitfield (1974) using the equation; $\% \text{UIA} = 100 [1 + \text{antilog} (\text{pK}_a^\circ - \text{pH})]^{-1}$, was similar to that obtained by Epifanio & Srna (1975), although their experimental conditions resulted in a greater range (1.5-2.8% compared to 2.01-3.0% in this study). These figures produced lethal concentrations for *M. mercenaria* and *C. virginica* respectively, of 1.65-3.08 ppm and 13.2-24.6 ppm NH_3 . Comparison with this study and previously published toxicity values suggest an unusually high tolerance in these species or a concentration overestimate as discussed earlier. The acute toxicity level for *Pecten maximus* falls within the range 0.63-1.00 ppm NH_3 .

The toxicity of ammonia in an aquatic system is clearly dependent on the physico-chemical conditions which prevail. How then does this relate to the measured haemolymph and mantle fluid concentrations determined in this study? The concentration of haemolymph ammonia in *Pecten maximus* increased during a 48 hour emersion from 17.1 to 448.6 $\mu\text{mol.l}^{-1}$ (7.6-8.1 ppm total ammonia for the 48 h value), but over the same time period in an equivalent sample, blood

pH decreased from 7.6 to 6.87. The result of this would be that the NH_3 fraction would reach only $0.49 \mu\text{mol.l}^{-1}$ (0.009 ppm), instead of $2.56 \mu\text{mol.l}^{-1}$ (0.045 ppm) had the pH remained constant. Thus, internal toxicity will be within the sub-lethal range rather than the acute. In contrast, the ammonia concentration of the mantle fluid, which reaches a similar level of $413.6 \mu\text{mol.l}^{-1}$ (7.03-7.44 ppm total ammonia) after 48 hours, will tend to have a more toxic effect due to the smaller change in pH. In this fluid, pH after 48 hours was 7.26 giving an NH_3 concentration of $1.07 \mu\text{mol.l}^{-1}$ (0.02ppm). Whilst both these values are within the sub-lethal toxicity range, certain other factors may alter the proportions or effects of un-ionised ammonia. For example, decreased temperature will reduce the NH_3 component which may be relevant in chilled transport conditions, and, as mentioned previously, the reduced oxygen concentrations of blood and mantle fluid may increase the toxic effect of the ammonia, without actually changing its relative proportions.

Even without these factors, however, measured NH_3 concentrations are probably sufficient to cause sub-lethal effects after 48 hours and it would seem likely that ammonia accumulation will continue throughout the period of emersion. Higher NH_3 concentrations were found in the mantle cavity, which is of particular importance since one of the recognised effects of ammonia on aquatic organisms is damage to gill tissue. During emersion the gills of the scallop, lacking the usual support of water, tend to lie in the remaining mantle-cavity fluid, and it has been noted that gill epithelia are particularly susceptible to ammonia, even in extremely low concentrations. Burrows (1964) reported hyperplasia and 'clubbing' of the gill filaments of salmonids at NH_3 concentrations less than 0.01ppm, and Jeney *et al.* (1992) found evidence of tissue necrosis, including gill epithelial tissue, in carp exposed to 0.34 ppm.

The actual processes resulting in tissue necrosis have not been firmly established, although a number of mechanisms have been proposed. Both forms of ammonia can cause different pH effects dependant on the concentrations of each, and which form is being transported across cell membranes. Ionised ammonia, though thought to be the less permeable fraction, is weakly acidic, whereas NH_3 is strongly basic. If sufficient quantities of ammonia are present then the resultant pH changes may impair cellular function (Campbell, 1991) and lead to cell death. Additionally, NH_4^+ permeability of cell membranes is linked to its substitution into the $\text{K}^+\text{-Na}^+$ active transport mechanism and it has been suggested that at low pH, when NH_4^+ predominates, ionic imbalance of the cell may occur due to competitive exclusion of Na^+ , again resulting in functional deterioration and necrosis (Shaw, 1960; Armstrong *et al.*, 1978). The evidence for this mechanism is, however, ambiguous (Young-Lai *et al.*, 1991) and awaits further clarification. Another proposed mechanism involves the translocation of NH_3 across cellular membranes which results in the binding of H^+ ions. This removes the H^+ gradient required for ATP production and effectively uncouples oxidative phosphorylation in mitochondria (Brierly & Stoner, 1970). Reduction of ATP levels has been reported during sub-lethal ammonia exposures

(Jeney *et al.*, 1992) and the resultant energy deficit may also cause cellular deterioration. With these potential effects on cellular function, it is likely that the general toxicity of ammonia is due to a combined action causing initial damage and perhaps enabling other factors to become significant.

The gradual deterioration of the gill tissue of *Pecten maximus*, when exposed to high concentrations of both NH_3 and NH_4^+ , is likely to be at least partially caused by the presence of the ammonia, since the macroscopic structural breakdown was also observed in the absence of a large bacterial population during the toxicity experiments. However, some studies have highlighted the importance of the initial epithelial/cellular breakdown, due solely to ammonia effects, as facilitating the invasion of bacterial pathogens (Burrows, 1964; Jeney *et al.*, 1992). Long-term exposure to low levels of ammonia may therefore result in the occurrence of chronic disease processes, but exposure to relatively high ammonia concentrations, coupled with conditions favourable for bacterial proliferation, may cause rapid death in affected animals. Since such conditions undoubtedly prevail in this present situation, the contribution of bacteria to tissue damage cannot be ignored.

Introduction

There appear to be 2 major microbiological obstacles facing the development of the shellfish industry (West, 1989). Firstly, the effect of disease on cultured and natural stocks of shellfish and, secondly, the effect on consumer confidence of outbreaks of food poisoning and other illnesses caused by contaminated or poorly prepared seafood (Dupont, 1986; Pain, 1986). These problems may be caused by natural or introduced bacteria depending on the circumstances of culture and post-harvest treatment.

Pathogenic microorganisms affecting scallops appear to be quite rare and hence the culture of pectinids has largely escaped the disease problems associated with other aquaculture systems (Sindermann, 1970). Although pectinids suffer from few bacterial infections, some examples are known and have come to light largely as a result of experimentation with the production of hatchery larvae. Leibovitz *et al.* (1984) reported 3 bacterial diseases, including bacillary necrosis and vibriosis, which affected larval *Argopecten irradians* reared under hatchery conditions.

The main exception to this pattern is rickettsiosis, a relatively poorly described disease affecting adult *Pecten maximus* (Le Gall *et al.*, 1991), *Placopecten magellanicus* (Gulka *et al.* 1983) and *Argopecten irradians* (Morrison & Shum, 1983; Leibovitz *et al.*, 1984). Rickettsia-like microorganisms (*Rickettsia* being a genus of Gram negative, rod shaped bacteria) have been found in the gill epithelia of diseased and dead scallops and, while no specific cause of death has been identified, Gulka *et al.* (1983) suggested that gill dysfunction precipitated physiological and metabolic stress and ultimately led to pathological changes in soft tissues.

Many marine bivalves, including *Pecten maximus*, are benthic filter feeders and are likely to be contaminated with relatively large numbers of bacteria due to the concentrating effect of their feeding method. This factor becomes particularly important if sewage effluent is discharged into water in which the animals are feeding since it is here that most potential human pathogens are found. Microorganisms ingested in this way are not necessarily destroyed and may remain viable until consumed by humans (Jorgensen, 1966), with the resultant public health problems, or excreted in the faeces of the mollusc. As a consequence of this, many commercially harvested bivalves undergo a period of purification before marketing, during which the animals are supplied with clean or decontaminated water and left to expel their bacterial load naturally. This system, although widely used, may not completely resolve the problem of contamination since reinfection of clean shellfish is possible due to the persistence of viable microorganisms in expelled faecal material (Rowse & Fleet, 1982).

Pectinids have a natural bacterial flora inhabiting the surface layer of the animal which live commensally with their host under normal conditions. It is likely that this flora does not differ significantly from that of other marine bivalves. Colwell & Liston (1960) described the most common genera isolated from the Pacific oyster, *Crassostrea gigas*, which included; *Pseudomonas*, *Vibrio*, *Flavobacterium*, and *Micrococcus*. These bacteria are controlled by the host's natural defence mechanisms, such as ciliary movement and mucus secretion, and are completely excluded from some ciliated epithelia where uncontrolled growth could cause pathogenic infection (Garland *et al.*, 1982). It follows that, should these control mechanisms break down, the very same bacteria will multiply and lead to disease or spoilage (Shewan, 1971). It may be necessary, therefore, to apply bacterial control methods during transportation to market or ongrowing sites in order to minimise the risk to consumers and stock, should any of this flora be potentially pathogenic.

Microorganisms clearly represent an important feature of shellfish culture and marketing and their effects became apparent during the early stages of the present study. It was observed, that during respiration experiments in air, scallop mantle cavity fluid appeared cloudy after 2-3 days and this coincided with the appearance of putrefaction odours. After 5 days of emersion, areas of red and purple colouration appeared on the scallop muscle and gonad which were identified as colonies of *Serratia marcescens* (J. Murray, pers. comm.). Microscopic observation of these samples and fluid taken from the mantle cavity confirmed the presence of large numbers of bacteria which was correlated with the increase in the rate of oxygen consumption after approximately 80 hours of exposure (Fig. 4.1). Observations also showed that during this period the gill filaments of the scallops were separating and becoming eroded at the filament margins. This effect would clearly compromise the efficiency of the gill and perhaps lead to a more rapid death. It was therefore decided to identify the cause(s) of gill damage and determine what effect this had on scallop mortality. Dependent on this, bacterial control may improve scallop survival and appearance.

Initially, therefore, bacterial investigations such as colony counts and growth rates were carried out to explain unexpected respiratory data and experimental observations. When the extent of bacterial colonisation became clear it was necessary to make a more complete study with the intention of identifying the bacteria present, and linking their physiology and metabolism with observed detrimental effects on emersed scallops. Identification of bacterial taxa is largely dependant on the reaction of an isolate to a series of morphological, biochemical and physiological tests. The results of these tests, usually negative or positive reactions, are used in determinative manual (Buchanan & Gibbons, 1974) or flow diagram form (Oliver, 1982) to identify isolates to family or genus level. Species designation requires application of considerably more tests, and therefore generic identification is standard. If a feasible method of

bacterial control could then be determined, perhaps specifically directed towards the most important genera identified, then the subsequent reduction in detrimental bacterial effects may improve scallop condition and survival.

Maintenance of experimental animals

Scallops of shell height $10 \pm 1\text{cm}$ were obtained from the Sea Fish Industry Authority marine farming unit, Ardtoe, Argyll, and transported to the Department of Zoology, Glasgow University in double lined, water-filled polythene bags which had been sealed and inflated with oxygen. Upon arrival, the scallops were maintained in aquaria which were supplied with re-circulating sea water at a temperature of $10 \pm 1^\circ\text{C}$ and salinity of 32‰. Aquarium sea water, obtained from the Firth of Clyde, was replaced at quarterly intervals. Scallops used for microbiological analysis were used within 5 days of arrival.

5.1. Bacterial growth rates during emersion

Materials and method

Two scallops were placed in Perspex flasks (see section 4.2.) over a 7 day period at $10 \pm 1^\circ\text{C}$. Mantle cavity fluid samples of approximately $200\ \mu\text{l}$ were removed daily using sterile plastic pipette tips. Of this sample, $100\ \mu\text{l}$ was diluted with autoclaved sea water to produce concentrations estimated to give bacterial colony numbers of less than 100 per plate to facilitate counting (Table 5.1). Once diluted, $100\ \mu\text{l}$ of each sample was plated in duplicate on 90 mm petri dishes containing Difco marine agar 2216 and incubated at 20°C for 5 days.

Counts of bacterial colonies were made after 2 and 5 days of incubation and the results adjusted to take account of dilution factors. The final counts were expressed in colony forming units per ml of mantle cavity fluid (CFU ml^{-1}).

Results

The mean bacterial count from freshly-emersed scallops was $1.02 \pm 0.12 \times 10^4\ \text{CFU ml}^{-1}$ ($n=2$)(Table 5.1, Fig. 5.1). Over the following days of emersion bacterial numbers increased significantly (ANOVA, $P < 0.05$), reaching $3.30 \pm 0.62 \times 10^{11}\ \text{CFU ml}^{-1}$ after 7 days in air.

Fig. 5.1 *Pecten maximus*. Growth of bacteria in mantle-cavity fluid during a 7 day emersion at $10 \pm 1^\circ\text{C}$. Daily fluid samples plated on nutrient agar and bacterial counts made after 5 days incubation at 20°C . Values are means of replicate samples expressed in colony forming units per ml of mantle cavity fluid ($n=2$). Error bars omitted for clarity, see Table 5.1 for values. Data have been log transformed.

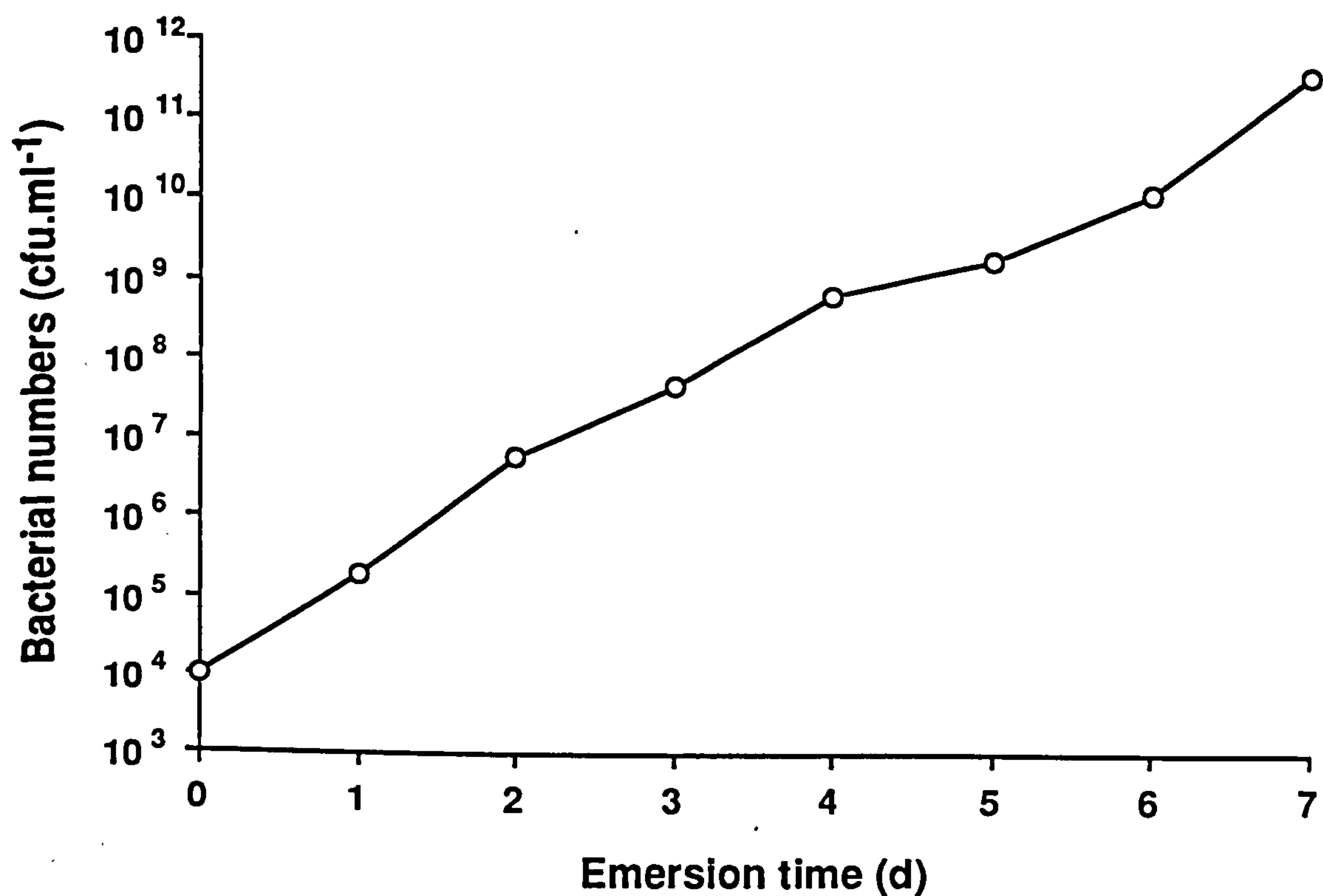


Table 5.1 *Pecten maximus*. Daily bacterial counts of fluid drawn from the mantle cavity during a 7 day emersion at 10 ±1°C. Values are mean counts (±S.D.) of two replicates (n=2) made after 5 days incubation at 20°C. Values are expressed in colony forming units per ml of mantle fluid (CFU ml⁻¹). Also shown are the dilution factors applied to mantle cavity fluid samples in order to produce suitable numbers of bacteria to facilitate counting.

DAYS EMERSION	DILUTION	Bacterial numbers (CFU ml ⁻¹)
0	1:10	1.02 ±0.1 x 10 ⁴
1	1:10 ³	1.95 ±0.3 x 10 ⁵
2	1:10 ⁴	6.05 ±2.2 x 10 ⁶
3	1:10 ⁵	4.65 ±1.1 x 10 ⁷
4	1:10 ⁶	6.15 ±1.1 x 10 ⁸
5	1:10 ⁶	1.74 ±0.3 x 10 ⁹
6	1:10 ⁷	1.14 ±0.9 x 10 ¹⁰
7	1:10 ⁷	3.30 ±0.6 x 10 ¹¹

5.2. Control of bacterial growth using antibiotics

Materials and method

Prior to exposure in air half of the scallops (n=4) were bathed in a solution of 0.03g.l⁻¹ Benzylpenicillin BP and 0.03g.l⁻¹Streptomycin sulphate BP (dissolved in sterile sea water) for a period of 90 minutes. The remaining animals were used as a control group. Both groups of scallops, treated and control, were emersed in perspex flasks within a water bath set at 10°C. The procedure of bacterial isolation was similar to that described in 5.1 except that the initial samples were diluted 1:10².

Results

Mantle cavity fluid bacterial numbers from both treated and control scallops over the experimental exposure period are shown in Fig. 5.2 and Table 5.2. Numbers were significantly different (2 sample t-test, P< 0.05) at the initial emersion reflecting the effect of the antibiotics. Both populations increased significantly over the following 7 days in air (ANOVA, P<0.05) and remained significantly different from each other until day 5 (2 sample t-test, P<0.05 between days 1-4). After this time bacterial numbers were similar.

Fig. 5.2 *Pecten maximus*. Growth of bacteria in the mantle-cavity fluid during a 7 day emersion at $10 \pm 1^\circ\text{C}$. Daily fluid samples plated on nutrient agar and bacterial counts made after 5 days incubation at 20°C . Closed symbols represent antibiotic-treated scallops, open symbols represent untreated controls. Values are means of replicate samples expressed in colony forming units per ml of mantle-cavity fluid ($n=2$ for each treatment). Error bars omitted for clarity, see Table 5.2 for values. Data have been log transformed.

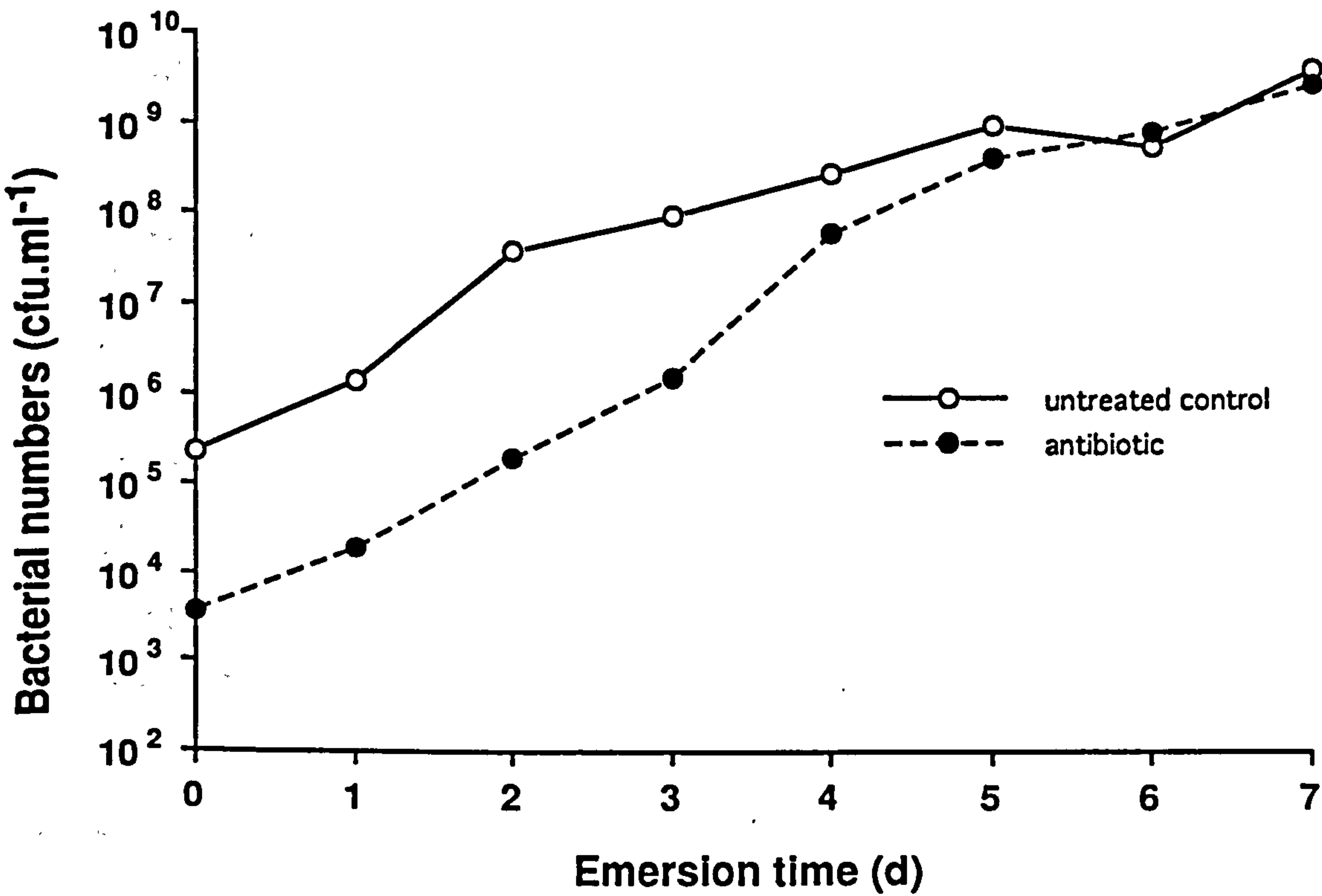


Table 5.2 *Pecten maximus*. Daily bacterial counts of fluid drawn from the mantle cavity during a 7 day emersion at 10 ±1°C. Scallops were untreated controls (n=2) or treated with antibiotics (n=2). Values are mean counts (±S.D.) of two replicates made after 5 days incubation at 20°C. Values are expressed in colony forming units per ml of mantle fluid (CFU ml⁻¹).

DAYS EMERSION	Control bacterial numbers (CFU ml ⁻¹)	Antibiotic bacterial numbers (CFU ml ⁻¹)
0	2.38 ±0.2 x 10 ⁵	3.75 ±2.3 x 10 ³
1	1.44 ±0.2 x 10 ⁶	2 ±1.4 x 10 ⁴
2	3.75 ±1.0 x 10 ⁷	2 ±2.0 x 10 ⁵
3	9.37 ±5.6 x 10 ⁷	1.5 ±1.2 x 10 ⁶
4	2.75 ±1.2 x 10 ⁸	6 ±4.9 x 10 ⁷
5	8.75 ±4.9 x 10 ⁸	4 ± 1.6x 10 ⁸
6	5.25 ±4.4 x 10 ⁸	8 ±4.5 x 10 ⁸
7	3.82 ±2.0 x 10 ⁹	2.52 ±1.6 x 10 ⁹

5.3. Effect of antibiotics on scallop survival

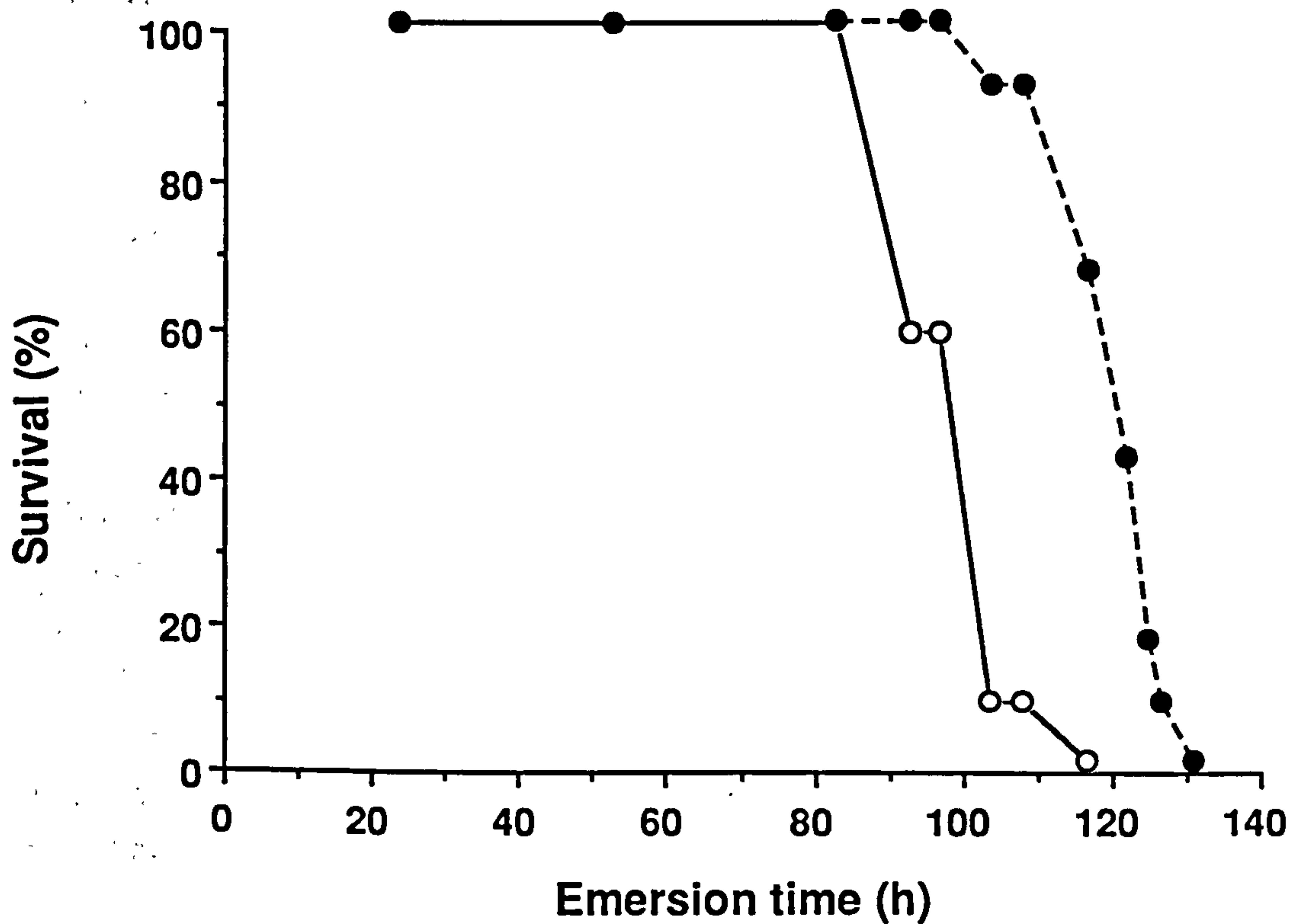
Materials and method

Experimental animals (n=12) were pre-treated with an antibiotic solution of 0.03g.l⁻¹ Benzylpenicillin BP and 0.03g.l⁻¹ Streptomycin sulphate BP (dissolved in sterile sea water) for 90 minutes prior to emersion at 10°C and 95 ±2% r.h. in the survival chamber apparatus described in 3.1. An equal number of scallops were emersed under the same conditions, but without the antibiotic treatment, to act as a control group. Assessment frequency and criterion of death were the same as those described in 3.2.

Results

The first deaths in the trial were recorded between 81 and 91 hours in the control group (Fig. 5.3). The first deaths in the antibiotic treated group occurred between 95 and 102 hours. Comparison of the times of equivalent % survival indicates a consistent difference of approximately 20 hours between groups throughout the experiment. The difference in maximum survival was 14 hours. The overall difference between the treatments was significant over the course of the trial (Kolmogorov-Smirnov 2 sample, 2-tailed test, P< 0.01).

Fig. 5.3 *Pecten maximus*. Survival during emersion at $10 \pm 1^\circ\text{C}$ and $95 \pm 2\%$ r.h. Closed symbols represent scallops treated with an antibiotic solution for 90 min. prior to emersion (see text for details). Open symbols represent untreated scallops. $N=12$ for both conditions. Difference in survival was significant using the Kolmogorov Smirnov two-sample, two-tailed test, $P<0.01$.



An interesting observation made throughout this experiment was that those scallops treated with the antibiotic solution retained a much cleaner internal appearance relative to the control group. Mucus production appeared to be reduced and mantle cavity fluid remained clear for a much longer period in comparison to the control group.

5.4. Bacterial isolation and identification

Materials and methods

Four scallops, 2 treated with antibiotics and 2 untreated controls, were emersed in Perspex flasks for 5 days at 10°C. After this time, mantle cavity fluid samples were taken, diluted to 1:10⁶ and plated in duplicate on Difco marine agar 2216. A nutrient agar plate was exposed to the air flow, which supplied the chambers during emersion, to act as a control in case airborne microbes were colonising the mantle cavity of the scallops. The agar plates were then incubated at 20°C for 3 days and the number of different colonies recorded.

Results

Plating and incubation procedures resulted in a total of 40 individual colonies. These were; 20 from control scallops (isolate numbers 1-18, 2 isolates were lost due to colony death), 15 from antibiotic-treated scallops (isolate numbers 19-33) and 5 from the air supply control (isolate numbers 34-38).

After these bacterial colonies had grown sufficiently, sample loops of each colony were removed and replated on the same growth medium in individual petri dishes, and a series of morphological and biochemical tests were carried out to allow identification of the bacterial genera. The following tests; colony characteristics and morphology, gram stain, motility, oxidation/fermentation, oxidase activity, acid and gas from glucose fermentation, catalase activity, O/129 sensitivity and luminescence, comprise a relatively standard identification procedure and were adopted following advice from the Department of Microbiology, Glasgow University. The complete results of these tests are shown in Tables 5.3 and 5.4.

5.4.1. Colony characteristics and morphology

Materials and method

Pigmentation, morphology and growth characteristics of bacterial colonies isolated on fresh nutrient medium constitute relatively simple, though important, taxonomic features. The colour, texture and size of each colony was assessed visually and recorded. These features were recorded after 5 days incubation at 20°C.

Results

Table 5.3 lists the main visual characteristics of each colony in detail.

Table 5.3 The visual characteristics of bacterial colonies isolated from the mantle cavity of scallops after 5 days emersion at 10°C. Isolates 1-18 from control scallops, 19-33 from antibiotic treated scallops, and 34-38 from the air supply.

ISOLATE NUMBER	COLONY COLOUR	COLONY TEXTURE	COLONY DIAMETER(mm)
1	YELLOW/ORANGE	SMOOTH	4
2	ORANGE	SMOOTH	2
3	CREAM	SMOOTH	4
4	BRIGHT YELLOW	SMOOTH	1
5	YELLOW	SMOOTH	2
6	Translucent Cream	SMOOTH	3
7	Translucent Cream	GRANULAR	2
8	YELLOW/ORANGE	SMOOTH	2
9	ORANGE	SMOOTH	1
10	ORANGE	SMOOTH	5
11	CREAM	SMOOTH	7
12	Translucent Cream	SMOOTH	6
13	PALE YELLOW	SMOOTH	2
14	PALE ORANGE	SMOOTH	8
15	CREAM	SMOOTH	6
16	Translucent Cream	SMOOTH	10
17	CREAM	SMOOTH	2
18	Translucent Cream	SMOOTH	6
19	ORANGE	SMOOTH	5
20	ORANGE	SMOOTH	4
21	Translucent Cream	SMOOTH	4
22	Translucent Cream	SMOOTH	4
23	Translucent Cream	GRANULAR	7
24	ORANGE	SMOOTH	3
25	Translucent Cream	SMOOTH	3
26	YELLOW/BROWN	SMOOTH	4
27	PALE ORANGE	SMOOTH	3
28	Translucent Cream	SMOOTH	7
29	Translucent Cream	SMOOTH	3
30	Translucent Cream	GRANULAR	4
31	YELLOW	SMOOTH	1
32	CREAM	SMOOTH	10
33	Translucent Cream	SMOOTH	4
34	ORANGE	SMOOTH	1
35	ORANGE	SMOOTH	2
36	Translucent Cream	SMOOTH	2
37	Translucent Cream	SMOOTH	2
38	CREAM	SMOOTH	2

5.4.2. Gram staining

Materials and method

This staining procedure differentiates bacterial species based on their cell wall structure and, additionally, allows classification as a rod shaped bacillus or spherical coccus (Atlas, 1986). A colony was taken from each original plate and grown for 24 hours on fresh marine agar. A loop of this fresh culture was then emulsified on a glass microscope slide in a drop of distilled water. The bacteria were fixed to the slide by passing it briefly through a bunsen flame. A drop of filtered, 0.5% crystal violet solution was placed on the colony for 1 minute. After rinsing in tap water a drop of iodine solution (20g potassium iodide (KI) dissolved in 250 ml distilled water to which was added 10g iodine before making up to 1 litre with distilled water) was added for 1 minute, rinsed again, and followed by a brief exposure (1 second) to a decolouriser solution (50% absolute alcohol and 50% acetone). Finally, the slide was treated for 30 seconds with a carbol fuschin solution (1g basic fuschin dissolved in 10 ml absolute alcohol mixed with 100 ml 5% phenol solution in distilled water and used in a 1:9 dilution with distilled water).

The slides were then examined using light microscopy with a x100 oil-emersion objective and the bacterial colour and shape recorded.

COLOUR	GRAM STAIN
Blue / Purple	+
Pink	-

Results

Of 38 isolates, 33 were identified as gram negative rods (-R), 4 as gram positive cocci (+C) and 1 gram positive rod (+R). Control scallops produced 16 gram - ve rods and 2 gram + ve cocci, antibiotic treated scallops produced 14 gram - ve rods and 1 gram +ve coccus and the air supply provided 3 gram - ve rods, 1 gram +ve rod and 1 gram +ve coccus.

5.4.3. Motility

Materials and method

Isolates were transferred, aseptically, into 10 ml bijoux bottles containing 5 ml of Difco marine broth 2216 and incubated overnight at 20°C. A loop of broth was collected, placed on a coverslip and inverted over a microscope cavity slide. The liquid was examined under a x40 objective and motility was recorded.

Results

Of 38 isolates, 29 were motile. There were 6 non-motile bacteria from control scallops, 2 from antibiotic treated scallops and 1 from the air supply. All gram positive cocci were non motile. Care was taken to ensure correct designation of motility since all bacteria oscillate when viewed under a microscope due to the effect of Brownian movement.

5.4.4. Oxidative/fermentative test

Materials and method

Bacterial metabolism may occur in the presence or absence of oxygen. The metabolism of oxidative bacteria, or aerobes, which involves oxidative phosphorylation, is dependant on the presence of oxygen. Some anaerobic bacteria, facultative anaerobes, are capable of both oxidative and fermentative metabolism, the latter occurring in the absence of oxygen. Obligate anaerobes employ only fermentative metabolism and are inhibited by the presence of oxygen. This test uses a chemical indicator to determine the broad nature of bacterial metabolism.

Difco Hugh and Leifson medium (M O/F medium; 200g O/F medium, 1.88g Basal medium, 5g NaCl) was prepared according to the manufacturer's instructions. A glucose solution was added aseptically to the medium via a 0.2 μ m acrodisc filter (Gelman Ltd.) to produce a total medium glucose concentration of 15%. The prepared medium was then dispensed into capped glass tubes, 3 ml per tube and 2 tubes per isolate. Both tubes were stab inoculated with each isolate and 1 ml liquid paraffin added to one of the tubes (anaerobic condition). The tubes were incubated at 20°C for 14 days, although regular checks were made throughout this period. After this time the reaction was recorded.

Oxidative / fermentative test reactions

REACTION		DESIGNATION
tube 1 (aerobic)	tube2 (anaerobic)	
yellow	yellow	fermentative
yellow	no reaction	oxidative
red	no reaction	alkaline
no reaction	no reaction	inert

Results

Of 38 isolates, 16 exhibited fermentative metabolism (6 control isolates, 7 antibiotic isolates and 3 air supply isolates), 2 exhibited oxidative metabolism (1 each from control and antibiotic sources) and 20 showed an inert reaction (11 control, 7 antibiotic treated and 2 air supply isolates).

5.4.5. Oxidase test

Materials and method

This assay tests for the enzyme oxidoreductase, indicating the presence of bacterial cytochrome c. A positive result is detected in the presence of the enzyme which rapidly catalyses the oxidation of tetramethyl-p-phenylenediamine into a coloured product. Cytochrome c is frequently lacking in facultative bacteria which are capable of living in aerobic and anaerobic environments.

Individual colonies were picked from the nutrient agar surface using thin wooden sticks and placed on to filter paper. A solution of tetramethyl-p-phenylenediamine (1% solution), prepared immediately beforehand, was added to each isolate and the resultant colour recorded.

Reactions for oxidase test

Colour	Reaction
purple, immediately or < 5 sec.	+
no colour or after > 3 min.	-

Results

Of 38 isolates, 25 exhibited an oxidase positive reaction (11 control, 10 antibiotic and 4 air supply isolates), 11 showed no oxidase activity (6 control, 4 antibiotic and 1 air supply isolate). Two isolates, 1 each from the control and antibiotic sources, showed no discernible reaction.

5.4.6. Acid and gas from glucose

Materials and method

Energy production from carbohydrate results in acid products, which may be buffered depending on the metabolism of the bacteria. Some bacterial groups are capable of further catabolism of the acid products into simpler compounds including CO₂ and H₂. Bubbles of gas produced as a result of these additional reactions allow further division of bacteria into acid or acid & gas producers.

Phenol red broth was prepared by dissolving 10g peptone (protease or pancreatic digest of casein) and 0.018g of phenol red indicator in 1 l of distilled water. Sodium chloride was added to produce a solution of 2% NaCl (total concentration) and the pH adjusted to 7.4.

The Phenol red broth solution was autoclaved in capped glass tubes, which contained small, inverted Durham tubes (care was taken to ensure that no air was trapped in these smaller tubes), at 15 p.s.i. for 15 min. and allowed to cool slightly. A 10% glucose solution (or other carbohydrate) was then sterilised by injection through a 0.2µm acrodisc filter into the phenol broth to produce a final concentration of 1% glucose. The prepared tubes were then aseptically inoculated with isolates and incubated at 20°C for 14 days. During this time the tubes were checked regularly and the medium colour and presence or absence of gas within the Durham tubes was recorded.

Colour	Reaction
pink	alkali
yellow	acid production

Results

After 14 days incubation 24 isolates showed alkali production (12 control, 7 antibiotic and all 5 air supply isolates). Metabolic acid production from glucose was shown by 5 of the control isolates and 6 antibiotic isolates. The 2 remaining antibiotic isolates and 1 control isolate showed no reaction due to absence of growth in the culture medium. No isolate exhibited gas production.

5.4.7. Catalase activity

Materials and method

This assay tests for the presence of the catalase enzyme which catalyses the decomposition of hydrogen peroxide (H_2O_2) into water and oxygen. Hydrogen peroxide is potentially toxic to microorganisms on account of its high oxidative capacity leading to disruption of phospholipid membranes. Consequently, bacterial groups have defensive mechanisms to break down such oxidisers, with catalase being present in aerobes and facultative bacteria (see 5.4.5).

Isolates were transferred, aseptically, onto Difco 2216 agar slopes prepared in bijoux bottles and incubated at 20°C for 24 hours. After this time 1-2 drops of hydrogen peroxide were added to the colony and the presence or absence of gas bubbles was noted. If gas bubbles were produced then the isolate was considered catalase positive.

Results

Catalase activity was shown by 31 isolates, of which 15 were from the control scallops, 14 were from antibiotic treated scallops and 5 from the air supply. Three isolates from control scallops and 1 from antibiotic treated scallops showed a negative response.

5.4.8. O/129 sensitivity

Materials and method

This test determined the sensitivity of each isolate to the compound O/129, an antibiotic which is specific to *Vibrio* species. The isolates were grown overnight in broth culture (see section 5.4.3), after which 100µl samples were transferred aseptically on to marine agar plates. The inoculum was spread over the medium surface and a 150µg O/129 disc (Oxoid DD15) was

placed on to the middle of the plate using sterile tweezers. The plates were then incubated for 48 hours and the growth of each isolate was recorded, a clear area around the disc, indicating absence of growth, was recorded as O/129 sensitive.

Results

No isolate exhibited a conclusive sensitivity to the presence of O/129 antibiotic. Possible sensitivity was shown by 3 isolates from the antibiotic treated scallops (isolate numbers; 19, 20, 27).

5.4.9. Luminescence

Materials and method

Colonies were removed from the original isolate plates, which had been stored at 3°C to retard further growth, and replated on to fresh Difco 2216 agar. These plates were then incubated for 24 hours at 20°C to allow new colonies to grow which were then observed in a darkened room to determine the presence of luminescent activity. The results of these observations were noted.

Results

No isolate exhibited luminescent activity.

Table 5.4 *Pecten maximus*. Detailed results of the various taxonomic and biochemical tests carried out to identify the bacterial isolates obtained from mantle-cavity fluid. Isolates 1-18 were obtained from control scallops, 19-33 from antibiotic treated scallops and 34-38 from the chamber air supply. (+) symbol refers to a positive result, (-) refers to a negative result and (o) refers to an inert reaction. Full taxonomic names, used in abbreviated form in this table, may be found in Table 5.5. Taxonomic names in bold represent more likely designations.

ISOLATE	GRAM STAIN	MOTILITY	OXIDASE	PIGMENT	OXIDATIVE
1	- R	+	+	+	O
2	- R	+	+	+	
3	- R	-	-	-	O
4	+ C	-	-	+	O
5	- R	-	-	+	O
6	- R	+	+	-	O
7	- R	-	+	-	O
8	- R	-	+	+	O
9	- R	+	+	+	O
10	- R	+	+	+	
11	- R	+	-	-	
12	- R	+	+	-	
13	+ C	-	-	+	O
14	- R	+	+	+	O
15	- R	+	O	-	+
16	- R	+	+	-	
17	- R	+	+	-	
18	- R	+	-	-	O
19	- R	+	+	+	
20	- R	+	+	+	
21	- R	+	+	-	O
22	- R	+	-	-	O
23	- R	+	+	-	O
24	- R	+	-	+	
25	- R	+	O	-	
26	- R	-	+	+	O
27	- R	+	+	+	
28	- R	+	+	-	
29	- R	+	+	-	O
30	- R	+	+	-	O
31	+ C	-	-	+	O
32	- R	+	+	-	
33	- R	+	-	-	+
34	+ C	-	-	+	O
35	+ R	+	+	+	O
36	- R	+	+	-	
37	- R	+	+	-	
38	- R	+	+	-	

Table 5.4 contd.

ISOLATE	FERMENTATIVE	CATALASE	ACID	ALKALI	GENUS
1	O	+		+	Pseud iii / iv
2	+	+	+		Vib / An Aer
3	O	+		+	Acinetobacter
4	O	+		+	Micrococcus
5	O	-		+	Cytophaga
6	O	+		+	Pseud iii / iv
7	O	+		+	Pseud / Mora
8	O	-		+	Cytophaga
9	O	+		+	Flavobacter
10	+	+		+	Aeromonas
11	+	+		+	Pseud / Alcal
12	+	+	+		Aeromonas
13	O	+	+		Micrococcus
14	O	+		+	Flavobacter
15		O	O	O	Ps.i/ii / Agro
16	+	+	+		Vib / An Aer
17	+	+		-	Vib / Aerom
18	O	+	+		Pseud / Alcal
19	+	+		+	Vib / An Aer
20	+	+	+		Vib / An Aer
21	O	+		+	Pseud iii / iv
22	O	+	O	O	Pseud / Alcal
23	O	+		+	Pseud iii / iv
24	+	+	+		Enterobacter
25	+	O	O	O	Enterobacter
26	O	+	+		Flavobacter
27	+	+		+	Vib / An Aer
28	+	+	+		Aeromonas
29	O	+		+	Pseud iii / iv
30	O	+		+	Pseud / Alcal
31	O	+		+	Micrococcus
32	+	+	+		Aeromonas
33		+	+		Pseud / Alcal
34	O	+		+	Micrococcus
35	O	+		+	Flavobacter
36	+	+		+	Vib / An Aer
37	+	+		+	Vib / An Aer
38	+	+		+	Vib / An Aer

Table 5.4 contd.

ISOLATE	FERMENTATIVE	CATALASE	ACID	ALKALI	GENUS
1	O	+		+	Pseud iii / iv
2	+	+	+		Vib / An Aer
3	O	+		+	Acinetobacter
4	O	+		+	Micrococcus
5	O	-		+	Cytophaga
6	O	+		+	Pseud iii / iv
7	O	+		+	Pseud / Mora
8	O	-		+	Cytophaga
9	O	+		+	Flavobacter
10	+	+		+	Aeromonas
11	+	+		+	Pseud / Alcal
12	+	+	+		Aeromonas
13	O	+	+		Micrococcus
14	O	+		+	Flavobacter
15		O	O	O	Ps.i/ii / Agro
16	+	+	+		Vib / An Aer
17	+	+		-	Vib / Aerom
18	O	+	+		Pseud / Alcal
19	+	+		+	Vib / An Aer
20	+	+	+		Vib / An Aer
21	O	+		+	Pseud iii / iv
22	O	+	O	O	Pseud / Alcal
23	O	+		+	Pseud iii / iv
24	+	+	+		Enterobacter
25	+	O	O	O	Enterobacter
26	O	+	+		Flavobacter
27	+	+		+	Vib / An Aer
28	+	+	+		Aeromonas
29	O	+		+	Pseud iii / iv
30	O	+		+	Pseud / Alcal
31	O	+		+	Micrococcus
32	+	+	+		Aeromonas
33		+	+		Pseud / Alcal
34	O	+		+	Micrococcus
35	O	+		+	Flavobacter
36	+	+		+	Vib / An Aer
37	+	+		+	Vib / An Aer
38	+	+		+	Vib / An Aer

5.4.10. Taxonomic method

Materials and method

After the results of each test had been collated for all isolates, the following identification schemes were used to identify the bacteria; Buchanan and Gibbons (1974), Oliver (1982), Muroga *et al.*, (1987). These methods employ a simple flow chart scheme which determines the generic allocation depending on the results from each test. Results for every test were not obtained from each isolate and it was necessary to refer to several taxonomic schemes in order to reach a designation for all isolates. In the few cases where designations were still ambiguous after the 3 schemes had been applied, reference was made to the Department of Microbiology, Glasgow University, for suggestions as to the most likely genera.

Results

Of the 38 bacterial colonies isolated, the most common genera were *Vibrio* and *Aeromonas* constituting 34% of the total (Table 5.5). Many species belonging to these genera are difficult to differentiate without recourse to more complicated tests and it is therefore common practice to combine similar species together as *Vibrio/Aeromonas*. The next most common groups, both with 13% of the total, were the *Pseudomonas/Alcaligenes* complex and *Pseudomonas* groups iii & iv. The genera *Flavobacterium* and *Micrococcus* each comprised 11% of the isolates, and the family Enterobacteriaceae made up 5.5% of the total. *Cytophaga* species also represented 5.5% of species, with the remaining 8% of the total coming from the genera *Acinetobacter*, *Pseudomonas / Moraxella* and *Pseudomonas* group i / ii / *Agrobacterium*.

Table 5.5 *Pecten maximus*. Taxonomic designations of bacteria isolated from mantle-cavity fluid. Groups marked with an asterisk (*) had representatives in the air supply group.

BACTERIAL TAXA	Number of species isolated
<i>Vibrio / Aeromonas</i> *	8
<i>Aeromonas</i>	5
<i>Pseudomonas / Alcaligines</i>	5
<i>Pseudomonas</i> group iii / iv	5
<i>Flavobacterium</i> *	4
<i>Micrococcus</i> *	4
Enterobacteriaceae	2
<i>Cytophaga</i>	2
<i>Acinetobacter</i>	1
<i>Pseudomonas / Moraxella</i>	1
<i>Pseudomonas</i> group i / ii / <i>Agrobacterium</i>	1

5.5. Ciliostatic toxin and proteolytic enzyme assays

Observations made during experimental emersions indicated that gill and mantle tissue was subject to considerable structural breakdown which increased with the duration of the exposure. Associated with this was increased viscosity of the mantle cavity fluid and production of spoilage odours. Subsequent work indicated a significant bacterial increase, a probable explanation for the tissue degradation, since bacterial nutrition is largely dependent on the hydrolysis of available macromolecules by the secretion of exoenzymes into the surrounding medium. (Davis *et al.*, 1980). These enzymes are used primarily on dead organic material, partly because living organisms control bacterial numbers, thus preventing significant enzyme accumulation. However, stressful environmental conditions, such as emersion of sub-tidal bivalves, may lead to inefficient bacterial control or the proliferation of pathogenic bacteria which actively invade healthy tissue. Once tissue invasion begins in a stressed animal, particularly species with a limited immunological response, deterioration accelerates as bacteria proliferate in the nutrient-rich medium.

Bacterial exoenzymes include proteases, peptidases, polysaccharidases, mucopolysaccharidases, nucleases and lipases. These exoenzymes have been associated with bivalve mortality, particularly larval stages (DiSalvo *et al.*, 1978, Brown & Roland, 1984), and therefore may also be significant in tissue degradation and mortality of emersed scallops.

Protease activity of scallop flora was therefore investigated, with particular emphasis on ciliostatic enzymes (Nottage & Birkbeck, 1986, 1987a), a potential cause of gill damage and reduced respiratory function, since ciliary activity is a factor in the prevention of bacterial settlement and is directly responsible for water circulation over the gill surfaces.

Materials and methods

Bacterial isolation

An individual scallop was removed from water and exposed to air at $10 \pm 1^\circ\text{C}$ for 48 hours. A sample of mantle cavity fluid was then removed aseptically, diluted with autoclaved sea water to give concentrations of 1:10, 1:100 and 1:1000, and plated on to nutrient agar. Two nutrient media were used; Difco marine agar 2216 and Difco TCBS medium (Thiosulphate Citrate Bile Sucrose agar), the latter being specific for isolation of *Vibrio* species. *Vibrios* grow on both media, though only *vibrios* on TCBS. Initial plating was carried out using TCBS to ensure *Vibrio* designation, but, since 2216 medium isolates are easier to work with, the colonies were then transferred onto the standard medium.

Following incubation of the plates at 20°C for 72 hours, the most suitable dilution was determined, i.e. that which gave between 30 and 50 individual bacterial colonies per plate. A dilution of 1:1000 gave 46 '2216' isolates and 14 'TCBS' isolates.

Supernatant preparation

The isolated bacteria were grown at 20°C prior to inoculation into 50ml of Difco 2216 marine broth contained in 250 ml Erlenmeyer flasks. The flasks were incubated at 20°C and shaken at 100 oscillations min^{-1} for 72 hours. The broth was then centrifuged at 10,000 r.p.m. for 10 minutes and the resultant supernatant removed and stored at -20°C .

5.5.1. Ciliostatic toxin assay method

Sections ($\text{ca } 5\text{mm}^2$) of *Pecten maximus* gill were cut from a freshly dissected animal and placed in individual wells of a 5 x 5 well petri plate containing 1ml of sterile sea water (salinity 32‰) with an antibiotic concentration of 0.01% (Benzylpenicillin sodium BP and Streptomycin sulphate BP). Antibiotics were applied to prevent sample contamination during the experimental period. One ml of each experimental supernatant was added to each well to give a total volume of 2ml. Each supernatant was added in two forms; heat treated at 100°C for 5 minutes, to destroy heat labile toxins, and an unheated supernatant. Two controls were run comprising gill

sections exposed to 2ml of sterile, antibiotic-treated sea water and to 2ml of a 1:2 dilution of broth medium in sterile, antibiotic-treated sea water. All supernatants and controls were tested in duplicate. Plates were maintained at 20°C and examined at approximately 12 hour intervals for signs of ciliary activity. Observation of the lateral and latero-frontal cilia (Beninger *et al.*, 1988) of each gill section was made using a Leitz Diaplan light microscope.

Results

Ciliary activity in the gill sections was found to have ceased in all but one treatment (isolate 35) after 36 hours exposure to dilute supernatants. The time taken for ciliostasis in each gill section to occur ranged from 12 hours (isolates 39-42, 45-46) to 36 hours (isolates 3, 6-14). The supernatants from bacteria isolated on TCBS medium, 1-14, showed ciliostasis when examined at 19 hours. Little difference in ciliostatic effect was recorded between unheated and heated supernatants. Full results are shown in Table 5.6.

Control gill sections exhibited regular, co-ordinated ciliary beating up to 48 hours after dissection from the scallop, though some deterioration was evident in controls containing 50% nutrient broth.

Isolate 35 continued to show some ciliary activity up to the point when ciliary function in the broth controls began to decline. It was therefore impossible to differentiate between true supernatant effects and natural deterioration of the excised tissue.

5.5.2. Protease assay

Materials and methods

The protease activity of bacterial broth culture supernatants was assessed in two ways; visually and biochemically. At each microscopic examination for ciliary activity (see 5.5.3.), a visual estimate of gill structure breakdown was also made. This estimate was necessarily subjective but was useful in defining the nature and effect of bacterial action on gill tissue. The filament structure of the gill breaks down within 48 hours if the tissue is removed from the scallop and this process was observed to occur more rapidly in the presence of large numbers of bacteria. An estimate of the proteolytic activity of a given isolate was made by noting the extent of filament breakdown and assigning a value, in half point increments between 0 and 3, the highest value signifying complete gill breakdown and the lowest signifying an undamaged structure. The second, quantitative procedure, was based on the method described by Tomarelli *et al.* (1949) and Nottage *et al.* (1989).

Test fractions were used as 10 or 20 μ l samples and made up to 100 μ l with distilled water in Eppendorf vials. All experimental fractions were analysed in triplicate. To each sample was added 100 μ l of 1% azocasein solution (azocasein A2765, (Sigma Chemicals) in 0.1 M phosphate buffered saline, pH 7.5), the vials were then vortex-mixed before incubation at 37°C for 30 min. To sediment the remaining azocasein, 800 μ l of 5% TCA was added and the mixture centrifuged for 10 min. at 1000 r.p.m. From the resulting supernatant 500 μ l was removed and added to vials containing 500 μ l of 0.5 M NaOH. After vortex mixing, the absorbance of each sample was measured at 440 nm on a Philips PU 8620 UV/Vis. spectrophotometer.

A calibration curve, with Trypsin (T8003, Sigma Chemicals) as the standard protease, was determined using the method outlined above. Controls of uninoculated broth and distilled water were also carried out. Following correction due to dilution factors and the background value of the broth, the protease activity of each sample was determined from the curve and expressed as μ g trypsin equivalent, ml⁻¹ culture.

Results

The designations used for visually assessed proteolytic activity are illustrated in plates 5. 1-4.

Plate 5.1 (value 0) Undamaged gill section. Both principal and ordinary filament connections are intact giving a bright orange colour and regular cross-hatched pattern.

Plate 5.2 (value 1) The gill section is beginning to show breakdown of the filament connections, particularly at the descending/ascending tract junction, which gives the rather 'fuzzy' appearance. Microscopic examination of the tissue reveals erosion of the filaments and increased mucus production, resulting in cloudiness of the surrounding fluid.

Plate 5.3 (value 2) Gill sections exhibit even greater breakdown with the principal filaments showing as distinct rays. The ciliated ordinary filaments, which lie between the principal filaments, have almost completely broken down and the gill is beginning to fan out due to the loss of filament connections. Erosion of the filaments has continued and some have only the collagenous supporting rods remaining intact.

Plate 5.4 (value 3) Gill section completely broken down. The collagen rods of the filaments have been stripped of almost all cell layers and show no connections between them. Many of the ordinary filament rods have become completely detached. No cilia are visible under microscopic examination.

Plates 5.1-4 *Pecten maximus*. The effect of exposure to bacterial proteolytic enzymes on scallop gill tissue. Plates illustrate a progressive structural deterioration over approximately 48 hours and represent the standard breakdown values, from 0 (plate 5.1) to 3 (plate 5.4) used in Table 5.6. These standard values were used to qualitatively assess the proteolytic activity of supernatants derived from bacterial cultures. For further details see text. Magnification x12.

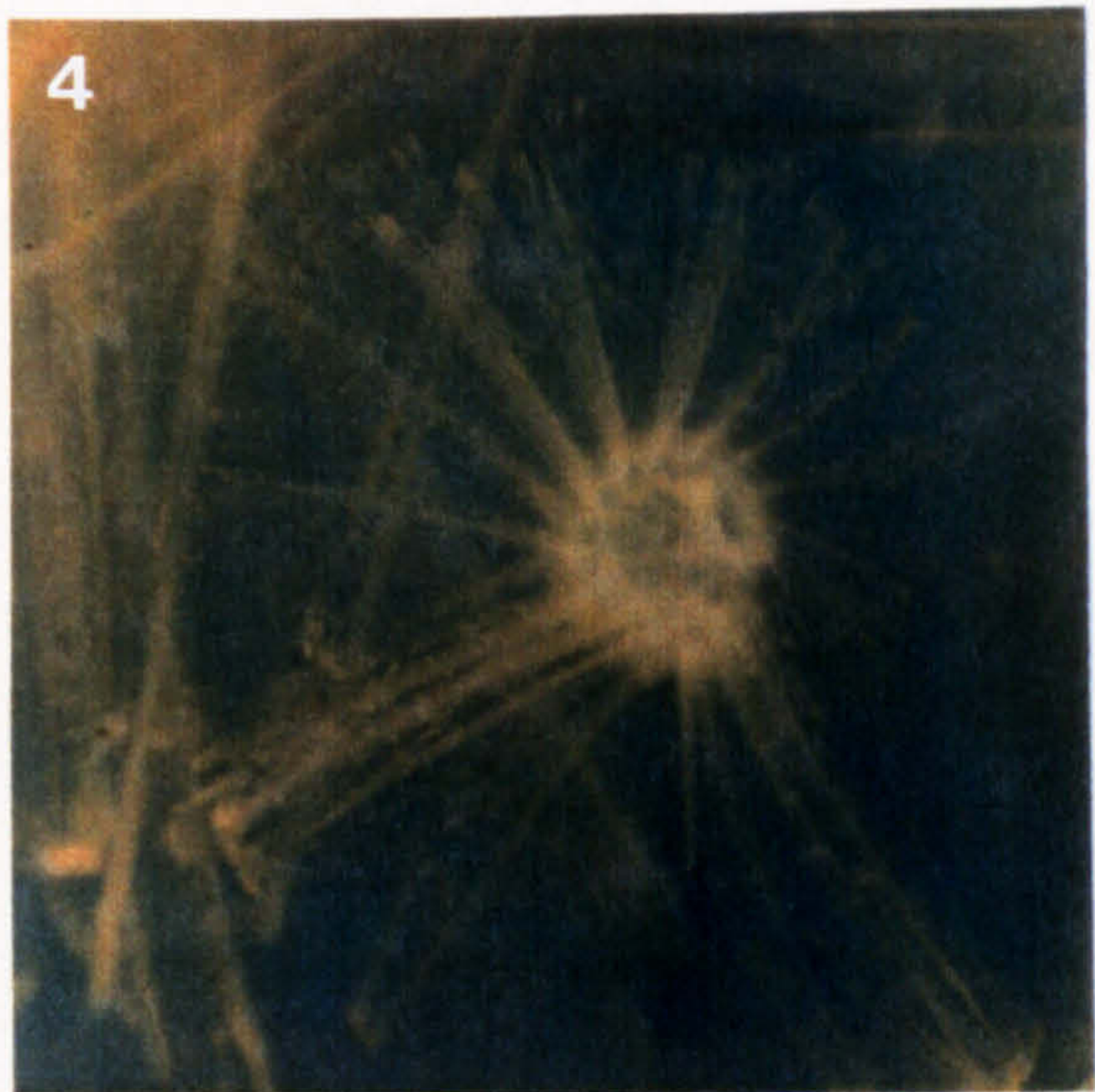
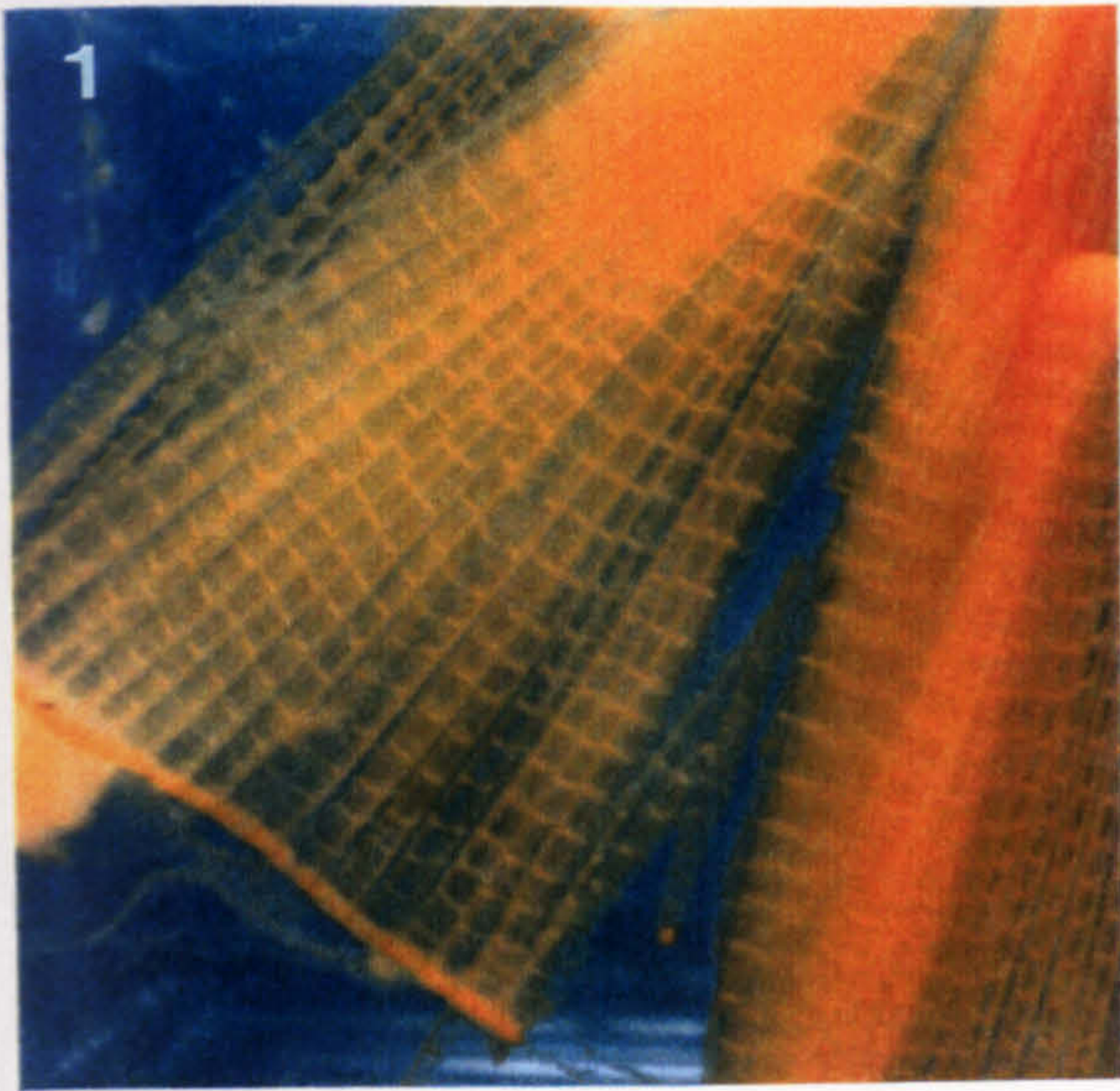


Table 5.6 shows estimated gill breakdown following a 48 hour exposure to a 1:2 dilution of untreated and heat treated supernatants. Since the values were not quantitative it was not possible to statistically compare the two treatments. However, in general, this qualitative assay estimated proteolytic activity to be higher in the untreated supernatants, suggesting the presence of a heat-labile component. Also shown in this table are the measured proteolytic values of normal supernatants, recorded as μg Trypsin equivalent per ml of supernatant. Recorded values of proteolytic activity ranged from $14.5 \mu\text{g Trypsin ml}^{-1}$ to $544.5 \mu\text{g Trypsin ml}^{-1}$. The minimum detection concentration of the assay was approximately $8 \mu\text{g Trypsin ml}^{-1}$ and measurements below this value could not be made. One isolate (35) was below this detectable level.

Table 5.6 *Pecten maximus*. The results of observational and biochemical assays to assess the ciliostatic and proteolytic activity in supernatants derived from broth cultures of the following bacterial isolates. Isolate numbers 1-46 represent mixed bacterial flora obtained from mantle-cavity fluid. Isolate numbers 1-14 (initially plated on TCBS medium) represent presumed *Vibrio* species obtained from the same source. N.D. indicates proteolytic activity was below the minimum sensitivity of the assay.

Isolate number	Time to Ciliostasis (hours)		Gill breakdown values after 48 hours		Proteolytic activity
2216 medium	Untreated	Heated	Untreated	Heated	$\mu\text{g Trypsin ml}^{-1}$
1	24	24	1.5	1.5	42
2	24	24	1.5	1.75	24.5
3	36	36	1.5	2	17
4	24	24	1.5	2	47
5	12	24	1.5	1.5	24.5
6	36	36	1.5	1.5	27
7	36	36	1.5	1.5	20.5
8	36	36	1.25	1.5	19.5
9	36	36	1.5	1.5	14.5
10	36	36	1.5	1.5	27
11	36	36	2	1.5	210
12	36	36	1.5	2	34.5
13	30	36	1.75	2	197.5
14	24	36	1.75	1.75	207
15	24	24	2	2	8.75
16	24	24	2.25	2	230
17	24	24	1.75	1.75	63.2
18	24	24	2.5	2	280
19	24	24	2	1.75	190
20	18	18	3	1.75	337.5
21	18	18	1.5	2	27
22	18	18	2.75	2	172.5
23	18	18	3	2.25	190
24	18	18	2.75	1.75	25
25	18	18	3	2	372.5
26	18	18	2	1	26.2
27	18	18	3	0.75	226.2
28	18	18	3	1	136.2
29	15	15	1.75	1.25	31.2
30	15	15	1.5	1.5	212.5
31	15	15	1.75	1.5	397.5
32	15	15	1.5	1.5	276
33	15	15	1	1.25	53.5
34	15	15	1.5	1.5	250

Table 5.6 continued

Isolate number	Time to Ciliostasis (hours)		Gill breakdown values after 48 hours		Proteolytic activity
2216 medium	Untreated	Heated	Untreated	Heated	$\mu\text{g Trypsin ml}^{-1}$
35	36+	36+	0.5	0.5	N.D
36	15	15	1.25	1.25	24.5
37	15	15	1.75	1.25	50
38	33	33	1.25	0.75	257.5
39	12	12	0.75	0.75	24.5
40	12	12	2.25	1.75	544.5
41	12	24	1	1	69.5
42	12	24	2.75	1.25	262
43	24	24	1.25	0.5	47
44	24	12	2.25	1.25	387
45	12	12	2.5	1	494.5
46	12	12	1.25	1	83.25
TCBS medium					
1	19	19	2	2	20
2	19	19	2.25	1.75	227.5
3	19	19	1.75	1	57.5
4	19	19	2	1.5	232.5
5	19	19	2	1.25	207.5
6	19	19	1.75	1.75	32.5
7	19	19	1.5	1.25	37.5
8	19	19	2.5	2	237.5
9	19	19	2.25	1.75	287.5
10	19	19	2	1.75	20
11	19	19	2	1.75	52.5
12	19	19	2	1.75	80
13	19	19	1.5	1.75	30
14	19	19	2	1.75	32.5

Discussion

The initial number of bacteria isolated from the mantle cavity fluid of emersed scallops was similar to numbers recorded from equivalent sources. Colwell & Liston (1960) recorded 1×10^4 bacteria per ml from the oyster, *Crassostrea gigas*, and Kueh & Chan (1985) estimated 5×10^5 bacteria per ml in the mantle cavity of the same species. This present study recorded 1.02×10^4 bacteria per ml of mantle fluid.

The increase in bacteria over the following 7 days of exposure was observed to have deleterious effects on the scallops. These included the typical signs of spoilage ranging from increased mucus production, cloudiness of pooled fluid, fading of tissue pigments, loss of flesh tone (particularly of the adductor muscle) and eventually to the presence of putrid odours. The rate and type of bacterial spoilage depends on a number of factors (Frazier & Westhoff, 1978).

- 1) Control mechanisms. Natural control mechanisms mediated by the animal or artificial mechanisms such as antibiotics.
- 2) Tissue type. Shellfish deteriorate quickly due to rapid oxidation of unsaturated fats.
- 3) Animal condition. Physiologically stressed, feeding animals with high intestinal bacterial counts deteriorate more rapidly.
- 4) Initial bacterial load. High initial bacterial counts tend to increase the rate of spoilage and therefore contamination of animals with mud during dredging will enhance deterioration.
- 5) Temperature. Higher temperatures encourage bacterial growth. Most bacteria from temperate waters are psychrophiles/psychrotrophs which can grow between -4 and 30°C with an optimum of between 20 and 25°C (Anon., 1981).

The natural bacterial control mechanism in bivalves involves ciliary movement which pumps a respiratory and feeding water current through the mantle cavity at a rate fast enough to prevent significant colonisation by microbes (Garland *et al.*, 1982). Those bacteria which do settle are trapped on a mucous layer which is constantly moved by the cilia, either into the gut, where digestion of the bacteria occurs, or expelled from the mantle cavity as pseudofaeces (Owen, 1966).

In the absence of a flushing water flow, as in the case of an emersed scallop, bacterial proliferation occurs and the processes of deterioration will commence. The most important spoilage microbes being those of the animal's natural flora (Colwell & Liston, 1960; Jay, 1978). Tanikawa (1937) identified the bacteria responsible for spoilage of the oyster, *Crassostrea gigas*, as belonging mainly to the genera; *Pseudomonas*, *Flavobacterium*, *Achromobacter* and *Micrococcus*. The results of identification procedures in this study confirm the presence of these genera (53% of the total) and most of the remainder, such as *Vibrio/Aeromonas* and

Cytophaga, while not directly implicated in spoilage, contribute to tissue breakdown in other ways.

Heterotrophic bacteria obtain nutrients from their environment primarily by hydrolysis of macromolecules such as proteins and lipids which originate from organic sources (Davis *et al.*, 1980). Hydrolysis is achieved by the secretion of exoenzymes, e.g. proteases and lipases, either into the medium, in the case of most Gram-positive bacteria, or into the periplasmic space (between the inner and outer membranes), in most Gram-negative bacteria. These enzymes, in addition to autolysis by any 'host' enzymes, act to break down proteins into peptides and amino acids, and possibly further to ammonia. Other nitrogenous compounds may be degraded to organic acids, ammonia and carbon dioxide.

Carbohydrates, such as glycogen, are preferentially utilised by microbes, since their hydrolysis usually results in readily usable monosaccharide sugars (Frazier & Westhoff, 1978). Glycogen is found in large quantities in the adductor muscle and gonad of scallops and therefore represents a potential nutrient source if invasion of the tissue occurs. The close proximity of the scallop intestine to the muscle and gonad, which it actually runs through, may facilitate invasion by bacteria already present in the animal.

If invasion occurs then epithelial membrane and tissue lipids may be degraded by microorganisms during spoilage reactions. Due to the unsaturated nature of most shellfish fats, however, auto oxidation is more common. This process results in oxidative rancidity and the breakdown products of fatty acids, glycerol, phosphate and nitrogenous bases. When bacteria are involved in fat breakdown the released lipases cause hydrolytic rancidity. Breakdown products are again fatty acids, glycerol or other alcohols which result in the characteristic and undesirable flavours and smells of decaying shellfish. These end products may also directly affect scallop tissue cells via osmotic and pH imbalance, leading to further necrosis and subsequent bacterial invasion.

Spoilage processes are generally brought about by aerobic organisms, but many marine bacteria are facultative anaerobes or fermenters whose activity results in putrefaction and the associated foul odours of hydrogen and methyl sulphides, amines and ammonia. Of particular importance in this respect are *Vibrio* and *Aeromonas* species which make up most of the remaining genera identified from this study. These genera are commonly isolated from shellfish (Vasconcelos & Lee, 1972; Kueh & Chan, 1985; Chan *et al.*, 1989) and particular importance may be placed on *Vibrio* species for 2 reasons. Firstly, their influence on the initial stages of soft tissue breakdown and resultant bacterial proliferation is important. As facultative anaerobes, *Vibrios* (and *Aeromonas*) are most abundant in the digestive tracts of marine shellfish and are usually the dominant genera there (Suzuki, 1977; Rodriguez & Hofer, 1986).

Under normal conditions ciliary activity is highly effective in controlling bacterial settlement within these tissues. For example, Colwell & Liston (1960) reported the complete absence of microbes on the intestinal surfaces, with any isolated bacteria coming from faecal or particulate matter within the gut. However, when emersed, scallops lack a flow of water to clear expelled material and therefore *Vibrio/Aeromonas* contamination of the mantle cavity will occur.

Additionally, *Vibrio* spp., are noted for the production of ciliostatic toxins (Nottage & Birkbeck, 1986; 1987a) which is likely to inhibit the passage of material through the gut and, hence, allow adhesion and proliferation of intestinal flora on the gut wall. Although ciliostatic activity by bacteria in relation to bivalve molluscs has been largely associated with *Vibrio* species (Birkbeck *et al.*, 1987), other genera are known to produce similar toxins, e.g. *Aeromonas*, *Moraxella* (Nottage *et al.*, 1989) and *Pseudomonas* (Reimer *et al.*, 1980; Hingley *et al.*, 1986a).

This study found ciliostatic activity in all but one of 60 bacterial isolates sufficient to cause complete cessation of ciliary movement on scallop gill within 36 hours. Although the isolates used in this experiment were not identified, with the exception of the *Vibrio* species, it is unlikely that they differed significantly from the 'typical' scallop flora classified earlier, since isolation techniques and growth media were identical. The 2 supernatant preparations from each isolate showed little variation in the timing of their ciliostatic effect. It is likely, therefore, that the majority of these ciliostatic toxins are heat-stable (Hingley, 1986a; Nottage *et al.*, 1989).

Thus, absence of a flushing water flow and the production of ciliostats may partially account for the observed increase in bacterial numbers during emersion. Once established, release of exoenzymes will begin to cause deterioration of body tissues. In a study of bacteria obtained from seafood and aquaculture products, Wong *et al.* (1992), reported that the enzyme activity of each species, mainly protease and lipase, was directly related to its cytotoxicity. It is probable therefore, that all but one of the bacteria isolated during this study will make some contribution to cell death and tissue breakdown. Bivalve gills are particularly susceptible to enzyme action and have been used as a qualitative assay for proteolytic activity (Nottage & Birkbeck, 1986; 1987b). Observations made during emersion experiments confirmed the delicate nature of scallop gills. This study found most bacterial supernatants had a significant effect on overall gill structure, though actual breakdown values varied from superficial damage to complete disaggregation of the filaments. Variation within each isolate, i.e. between untreated and heated supernatants, may be explained by the fact that some proteases are heat-labile (Jeffries, 1983; Brown & Roland, 1984) and their activity may be disrupted by treatment in a boiling water bath.

The destructive action on the gill appears to be concentrated initially on the ciliated epithelium and the ciliated discs, characteristic of filibranch species, which join the filaments and maintain the functional surface of the gill. Nottage *et al.*, (1989) suggested that there may be relationships between the different bacterial exotoxins such as the presence of proteases enhancing the effect of ciliostats (Hingley *et al.*, 1986a; 1986b). Breakdown of the filamentous structure of the gill and production of mucus in response to bacterial activity will have a profound effect on the efficiency of the tissue as a respiratory organ. Ciliostasis may result in a layer of deoxygenated water above the gas exchange surfaces, due to cessation of water currents, and the presence of dense aggregations of vesicles (Plate 4.9) on the filaments and circulatory vessels will further inhibit efficient gas exchange. Electron microscopy studies suggested that these vesicles probably represent the enclosed remnants of gill epithelial cells, indicating that the combined action of bacteria and scallop metabolites have already destroyed the gill as a functional tissue. An additional factor relating to oxygen uptake, prior to complete gill breakdown, is that such a large population of bacteria within the mantle cavity fluid, the medium from which much of the dissolved gases are exchanged, will reduce the fluid PO_2 during emersion and hence the amount of oxygen available for scallop respiration. Experimental results, detailed in chapter 4, have shown that mantle fluid oxygen concentration fell to 53% of the saturated value within 48 hours, and it is possible that lower values would result from longer emersions as the bacterial population increased.

The observed disaggregation of the gill filaments is an important feature of scallop emersion for a number of reasons. Firstly, the ciliated discs are presumably damaged by the toxic agents mentioned earlier and, once separated, have no means of rejoining. Disaggregation of the gill is therefore permanent. Bivalve sensitivity to these toxins appears greatest in juveniles and larval stages and therefore, while not directly related to marketing for consumption, there are great implications for the increasingly important practice of spat and juvenile transportation for ongrowing purposes. Brown & Roland (1984) reported an LC_{50} value for *Crassostrea virginica* larvae of less than $46.6 \mu\text{g}$ of *Vibrio* toxin l^{-1} of culture, a concentration produced by 2.9×10^9 bacterial colony forming units. This study recorded bacterial concentrations as high as 3.3×10^{11} , and, although of mixed genera with different toxin-production characteristics, these numbers must be considered significant. Experimental observations also suggest that filibranch species, such as *Pecten maximus*, may be more susceptible to toxin effects compared to bivalves such as *C. virginica* which possess a eulamellibranch gill, in which the filaments are connected by more substantial tissue junctions.

A further complicating factor is the apparently similar effects on the gill caused by high concentrations of ammonia. Disaggregation of gill filaments was recorded during measurements of ammonia accumulation in the blood and mantle cavity fluid and also during an ammonia toxicity trial carried out in water (see chapter 4), although this does not preclude a microbial

influence. The effects of this excretory product are dealt with more fully in the previous chapter, but epithelial erosion and gill damage are two recognised features of high ammonia concentrations. It is likely that bacterial exotoxin activity is enhanced by the presence of ammonia, either due to similar effects on tissue surfaces or by ammonia facilitating the invasion and colonisation of tissues by microorganisms.

In addition to the detrimental effects of bacteria on their 'host' organism, some microorganisms or their metabolic products can be important in terms of human health. This aspect is particularly significant in products such as shellfish which may be marketed as 'clean' and healthy foods but, by the nature of their physiology and habitat, need to be treated carefully if consumer confidence is to be maintained. The genera identified in this study include some important human pathogens, although it should be emphasised that the presence of such organisms does not necessarily result in clinical illness.

Vibrio and *Aeromonas* species are perhaps the most important and studied pathogenic bacteria associated with natural aquatic systems (Morris & Black, 1985). Nearly 50% of *Vibrio* species have been associated with human diseases (Tison & Kelly, 1984; Morris & Black, 1985) including *V. cholerae*, the cause of epidemic cholera, *V. parahaemolyticus*, a cause of gastroenteritis, and *V. vulnificus*, a cause of soft tissue and skin infections. All 3 species have been isolated from shellfish, which are considered to be important vectors for the bacteria (West, 1989).

Contamination by vibrios is dependent on the factors outlined above and in areas where environmental conditions are favourable, human activity is intensive and food handling is inadequate, serious outbreaks of disease can occur. *Vibrio parahaemolyticus*, for example, has been implicated in >60% of Japanese food poisoning cases, largely as a result of a high prevalence in the surrounding waters and the widespread consumption of raw fish (Aoki *et al.*, 1967). Chan *et al.* (1989) highlighted the prevalence of pathogenic vibrios in bivalve shellfish from Hong Kong markets, with numbers reaching 10^4 - 10^5 /g of tissue. An estimate of bacterial concentrations required to cause human illness is 10^6 - 10^7 /g of tissue (Sanyal & Sen, 1974), emphasising the need for proper handling, storage and cooking of shellfish. Outbreaks of *Vibrio*-related disease appears to be largely related to environmental temperature (De Paola *et al.*, 1990), which presumably controls numbers in the overlying waters. However, the presence of any such isolate implies potential risk if control methods are not applied.

Aeromonas species are also recognised as human pathogens (West, 1989), generally causing gastrointestinal illnesses in victims. A species of particular importance is, *A. hydrophila*, which has been associated with oyster-related gastroenteritis (Abeyta *et al.*, 1986).

The final group of directly important pathogenic bacteria identified in this study belong to the family Enterobacteriaceae. This family includes genera such as; *Salmonella*, *Shigella*, and *Yersinia* which are particularly important disease causing agents, although a more likely genus to be isolated from marine shellfish is *Enterobacter*, a coliform bacterium. This genus is important as a food spoilage group in its own right, but is often taken as an indicator of sewage contamination and therefore the possible presence of other enteric pathogens or viruses (Frazier & Westhoff, 1960).

Of the remaining identified taxa, most, including *Pseudomonas*, *Alcaligenes*, *Flavobacterium* and *Micrococcus*, are primarily enzyme producers and spoilage organisms and as such may cause human illness as a result of bacterial food intoxication, the ingestion of significant quantities of bacterial toxins. Again, quantities sufficient to cause illness occur only when large numbers of bacteria are present due to improper food storage and preparation.

From the preceding discussion it is clear that the human health risk posed by scallops or other bivalve shellfish obtained from clean, i.e. grade A (EEC, 1991), temperate waters relates more to treatment after harvest rather than to an inherent natural danger. It is, therefore, of greater importance to control bacterial numbers during transportation and preparation and thus avoid the majority of bacterial illnesses. Controlling the bacterial population would also ensure that the deleterious, and potentially lethal, effects of microbes on live shellfish are minimised. European community directives on bacterial numbers state that, prior to immediate consumption, 100g of mollusc flesh should contain no more than 300 faecal coliforms (e.g. *Escherichia*, *Enterobacter*) and no *Salmonella* should be present in 25g of flesh (EEC, 1991). These numbers must be considered conservative, since they relate to prevention of human illness after sale and consumption and, as such, may also represent an effective and attainable level for reducing the bacterial effects on the scallop. The regulations require means by which bacterial numbers can be controlled and this may be achieved by a number of methods (Silliker *et al.*, 1980);

- 1) Temperature
- 2) Antibiotics
- 3) Gases
- 4) Radiation
- 5) Acidity and pH
- 6) Smoking and salting
- 7) Packaging

In terms of the transport of live scallops only some of these methods are applicable, or indeed desirable, and it is these methods which will be discussed here.

Temperature, more specifically chilling, is the most commonly applied microbial control treatment applied to foods and it involves storage at temperatures close to, but above the freezing point of the food. In most cases this range lies between -1 and $+7^{\circ}\text{C}$, but the effectiveness of the technique depends largely on the temperature characteristics of the microorganism and the period of storage (Olson & Nottingham, 1980).

Bacteria fall into 4 main physiological groups in relation to temperature; thermophiles, mesophiles, psychrotrophs and psychrophiles. This classification relates growth performance to temperature and the majority of microbes isolated from temperate, marine waters, and hence from these scallops, are psychrotrophs. Morita (1975) defined psychrotrophs as cold-tolerant organisms with a maximum growth temperature above 20°C , but still capable of growth near 0°C . All isolates from this study were identified as belonging to taxa containing psychrotrophic species.

Chilling shellfish has the positive benefit of preventing the growth of most pathogenic bacteria since the majority are mesophiles and, hence, are below their minimum growth temperature under normal chilled conditions (Olson & Nottingham, 1980). Unfortunately, freezing would be required to prevent growth in psychrotrophic species, clearly undesirable for live shellfish, and therefore growth of these bacteria does occur during chilled transport. Although some of these species are patho/toxigenic and may be of potential risk to consumers (Schofield, 1992), the majority of psychrotrophs are spoilage organisms and therefore chilling does not prevent the processes of tissue deterioration. Altering the temperature within the range of chilling, -1 and $+7^{\circ}\text{C}$, merely acts to influence the dominant organisms, with pseudomonads, for example, predominating at lower temperatures (Tompkin, 1973). Pseudomonads, and certain other genera, do in fact continue to produce lipases and proteinases even down to 2°C (Alford *et al.*, 1971; Juffs, 1976). Such activity may cause further bacterial proliferation since it is known that psychrotrophs are stimulated by high concentrations of organic nutrients (Gounot, 1991) resulting from enzymic action.

Given the limitation of chilling, at least over long-term storage, Olson and Nottingham (1980) recommended that control measures to reduce the microbial population before chilling are important. In addition to this, the cooling process should be as rapid as possible to avoid large microbial populations developing during a slow decline through a range of optimum growth temperatures.

As mentioned previously, initial removal of some harmful bacteria from bivalves can be carried out at the point of harvest by self purification or depuration in clean water (Richards, 1988). This method is most often employed for bivalves harvested from sewage contaminated waters (West *et al.*, 1985), particularly those whose flesh is consumed entirely, e.g. mussels and oysters. Some concern has been voiced recently about the effectiveness of the technique with regard to human pathogens (Mesquita *et al.*, 1991), especially since viable bacteria can be excreted in the mollusc faeces only to re-contaminate other shellfish (Rowse & Fleet, 1982). Depuration is, however, a potentially useful technique for all shellfish since if performed properly, with decontaminated water and an appropriate flow rate, an overall reduction in microbial flora can be achieved.

Reduction in initial numbers of bacteria can also be achieved by treatment with antibiotics. Antibiotics act as bacteriostats, i.e. they reversibly inhibit growth, or as bactericidals, i.e. they have an irreversible lethal action (Davis *et al.*, 1980). The effectiveness of these substances was demonstrated in this study when overall bacterial numbers were reduced by approximately 10^2 -fold after the addition of 2 bactericidal compounds. Removal of bacteria had a positive effect on the overall survival of emersed scallops and additionally reduced mucus production within the mantle cavity.

Use of antibiotics has been associated with improved survival during transportation of other bivalve species, notably the giant clam, *Tridacna gigas*. Braley (1992) reported 98.5% survival of clams transported for a 30 hour period in the presence of streptomycin sulphate, compared with only 25% survival in an equivalent control. This work was carried out to improve the transportation of *T. gigas* to ongrowing sites rather than for consumption and therefore the main requirement was for live animals at the end of the trial. Addition of antibiotics for this purpose is likely to be the only feasible application for this present study because, although effective as food preservatives, the use of antibiotics in this regard is not encouraged due to the dangers of selecting resistant bacterial strains (Hurst, 1980). Specific legislation affecting the treatment of live shellfish with antibiotics in order to prolong transportation life appears to be lacking and recourse must be made to general laws concerning the antibiotic content of food. The American Food and Drug Administration did, however, sanction the use of a tetracycline dip of 5 p.p.m. for shucked scallop meat (Frazier & Westhoff, 1978), but the position in Great Britain is unclear. Antibiotics in relation to spat transportation will be discussed further in chapter 7.

The final method of bacterial control during transportation, applicable both to prepared foods and live bivalves, is gas treatment. Although many gases are used, or have potential use, as food preservatives (Clark & Takács, 1980), only carbon dioxide (CO_2) may be practical for scallop transportation. Dixon & Kell (1989), in a review of CO_2 control of bacteria, outlined the methods and effects of this treatment and made particular reference to seafoods. Bacteria may

be killed or inhibited depending on the concentration of gas used and the type of microorganism present. Important psychrotrophic spoilage genera such as; *Enterobacter*, *Flavobacterium* and *Micrococcus* are killed by exposure to 100% CO₂ at 25°C for 4 days (Coyne, 1932) and Pseudomonads were found to be inhibited at lower concentrations (Enfors & Molin, 1980). Carbon dioxide concentrations above 5% inhibit many food spoilage organisms (Clark & Takács, 1980) with a linear increase in inhibition up to 25-50% CO₂, depending on the food and flora involved (Ogilvy & Ayres, 1951). Ledward *et al.* (1971) and Clark & Lentz (1969) found that, in general, a 10% CO₂ concentration gave approximately 50% bacterial inhibition on the basis of total counts after a given incubation period.

The effectiveness of microbial inhibition by CO₂ also appears to be enhanced by a decrease in the storage temperature (Coyne, 1933; Clark & Lentz, 1969) and therefore a CO₂ concentration of 10-20% with a storage temperature of between 1 and 5°C may provide an effective control environment. The effect of such conditions on scallop physiology is unfortunately unknown and further investigation into this aspect may provide useful results in relation to the efficiency of CO₂ as a transport gas.

Chapter 6 Muscle physiology, adduction and gape

Introduction

The adductor muscles of the scallop are the most commercially important parts of the animal, particularly in relation to the live trade. The muscles are appreciated gastronomically as both a luxury and health food, mainly due to the high protein (50-90%) (Comely, 1974) and low lipid content (2.9-4.3%) (Taylor & Venn, 1979). Fresh scallop meat may retail at more than £16/Kg in Britain and up to 300FF (currently~£34)/Kg in France (Bamfield, 1991) and is therefore amongst the most expensive of fish products. The muscles may constitute as much as 57% of the flesh weight, and weigh more than 70g (Duncan, 1989), which also makes meat yield an important consumer feature of the scallop.

The general condition of the live scallop may be quickly assessed by the presence or extent of shell valve gaping, a feature directly controlled by the adductor muscles. The two adductor muscles are functionally separate and control different aspects of shell closure (Chantler, 1991). The large adductor is a striated muscle and is concerned with fast, repetitive closure of the valves, hence the name phasic adductor which refers to the muscle contraction type. The smaller adductor is a smooth muscle and, although involved to some extent in initial shell closure (Thompson *et al.*, 1980), its main function is to maintain closure with a prolonged or tonic contraction.

Valve opening is a passive action involving an external hinge ligament and an internal elastic ligament. The internal ligament, composed of the elastic protein abductin, is compressed as the muscles contract (Alexander, 1983) and, if adductor muscle tension is released for any reason, for example through cutting or fatigue, then the elasticity of the ligament forces the valves apart resulting in shell gape. It is assumed that degree of gape following emersion is related directly to the time since harvesting and, hence, to freshness. As a quality control feature of the live trade, gape must therefore constitute an important aspect of research, particularly the reasons for the apparent failure of the adductor muscles to control gaping during emersion.

Many other edible bivalves only begin to gape after death and, in comparison to the scallop, are able to survive for much longer emersion periods (Wijsman, 1976; Prochazka & Griffiths, 1991). For example, the oyster, *Crassostrea virginica*, can survive for six months in air (Medcof, 1959) while the scallop dies after only 3-4 days. It is of interest to know whether this survival difference is related to the timing of gape, perhaps as a result of differences in muscle metabolism during emersion which causes more rapid failure of scallop adductor muscles. In which case death may result from desiccation effects, which are enhanced with increasing gape (Coleman, 1973), or perhaps from physical stretching of the heart leading to cardiac failure.

Alternatively, gaping may indicate the onset of death which has been caused by another factor, particularly if this factor also affects muscle function and tension development. Initially, therefore, it was necessary to determine the relationship between gape and adduction and how these two parameters related to the duration of the emersion and to death, the time of which had been determined in previous experiments. This would indicate whether gape occurred before or after death and, additionally, might indicate how adduction activity is related to valve gaping.

Valve movements associated with escape swimming are thought to be based predominantly on anaerobic metabolism (de Zwann *et al.*, 1980) and, although earlier work showed that oxygen consumption is reduced during emersion, the two types of adduction may not necessarily use similar metabolic pathways. As a preliminary investigation into the muscle metabolism employed by the scallop, the general aerobic/oxidative capacity of the two muscles was investigated using histochemical-staining techniques. These techniques may indicate whether sustained aerobic metabolism was possible, even when oxygen uptake was reduced, or, as seems more likely, that anaerobic pathways are utilised. The main energy source for marine bivalves during emersion, whether good or poor survivors, is glycogen (de Zwann, 1983). If glycogen is used by *Pecten maximus*, then the rate or way in which it is metabolised may influence gaping and survival. Glycogen catabolism may produce variable amounts of energy, in the form of ATP, and different waste products dependant on the presence or absence of oxygen. In general, anaerobic metabolism produces less energy and more toxic waste products, and the ability to utilise anaerobic pathways efficiently and to tolerate the accompanying waste accumulation require specific biochemical adaptations. It seems unlikely that *Pecten maximus* will be well adapted for long-term environmental anaerobiosis, given that it is a wholly sublittoral animal, inhabiting oxygen rich areas of relatively high water movement. Since oxygen uptake is reduced during emersion, but activity remains high due to adduction activity, it is probable that the production of sufficient energy to meet metabolic requirements and the effects of waste-product accumulation will be important factors in sustaining individual tissues, such as the adductor muscles. Qualitative and quantitative biochemical assays have been used to determine whether or not glycogen is utilised as an energy substrate by *Pecten maximus* during emersion. In addition, the method by which ATP was produced from the energy substrate, either aerobically or anaerobically, was investigated.

If anaerobic metabolism is employed to any extent, then two potentially important factors must also be considered, those of end product and energy production. Sustained muscular function not only requires a continuous supply of ATP but also limited accumulation of the predominantly acidic waste products of anaerobiosis. Failure to meet these requirements will result in failure of the contractile mechanism and possible cellular disruption through pH or osmotic imbalance. As possible explanations for adductor failure and subsequent gape, as well

as being potential factors influencing long-term scallop survival, the concentrations of ATP and metabolic acids were investigated using high performance liquid chromatography (HPLC).

6.1. Recording of shell adduction frequency and shell gape during emersion

Materials and methods

The method used to measure shell movements was modified from the procedure described by Miyauti (1968). Individual scallops ($n=8$) used for recordings were placed in 10 l plastic aquaria which were connected to a sea-water input delivering water at 10°C. The experiments were carried out in a temperature controlled room which maintained air temperature at $10 \pm 1^\circ\text{C}$. Relative humidity was $95 \pm 2\%$. Before the aquaria were filled with water, the right (lower) valve of each scallop was forced into softened plasticine which was in turn attached firmly to the tank base. This prevented any horizontal movement of the animals during recording.

A length of thread was glued to the ventral margin of the left (upper) valve using gel-form cyanoacrylate adhesive. The tank was then filled and the scallops allowed to recover for 30 min. in gently aerated sea water. The free end of the thread was connected to an isotonic force transducer mounted on a retort stand. Adjustment of the apparatus position was made to maintain the transducer bar in a horizontal position when the scallop shell valves were closed. The transducer was connected to an oscillograph recorder (Washington MD2) via an AC coupler (Palmer Bioscience) which was also calibrated to the closed valve position. Thus, valve adduction changed the position of the transducer bar which was in turn converted into a pen trace to provide a continuous record of shell movement. Calibration of the transducer to allow measurement of shell valve gape, involved manual upward deflection of the transducer bar from the horizontal position over a 4 cm range, at 0.5 cm increments, the equivalent positions on the pen recorder trace were then marked. The transducer bar was connected to the upper valve of the scallop when the valves were tightly closed. The pen trace was returned to the zero calibration point by moving the transducer unit vertically on the retort stand. An increasing valve gape was recorded as a gradual deviation from this zero point which could be quantified. Following calibration and recovery, water was drained from the tank and recordings of adduction frequency and shell gape were made simultaneously. The scallops were emersed for periods up to 22 hours, by which time maximum valve gape had been attained and the reading had stabilised.

Results

When *Pecten maximus* is emersed, a series of between 10 and 20 rapid adductions occur within the first 3-4 min. The frequency of adduction then decreases over the following 10 min., to approximately 1 every 20 seconds, the shell valves being held about 1cm apart between adductions. The mantle edge, or velum, is held perpendicular to the shell margins at this time, forming a 'curtain' between the mantle cavity and the air. As the emersion progresses the mantle

edge withdraws from the shell margin and the velum falls back to lie on the mantle tissue itself, thus exposing the internal tissues. Adductions become less frequent and the distance between the shell valves slowly increases. The vellum appears to collapse when gape exceeds about 2cm.

The mean number of adductions for 30 minute periods during a 20 hour recording are shown in Fig. 6.1. Valve adduction commenced immediately upon emersion with a maximum frequency of 22.1 ± 9.2 (n=7) recorded during the first half hour. Adduction frequency then declined rapidly during the first 6 hours of exposure to only 1.3 ± 1.1 (n=7). Over the remaining 16 hours, adduction continued to decrease gradually with some scallops showing no shell movement for periods up to 5 hours. After 22 hours in air the mean adduction frequency was 0.5 ± 1.0 (n=4) per 30 minutes. The change in adduction frequency over the experimental period was significant (ANOVA, $P < 0.01$).

The changes in valve gape during emersion are shown in Fig. 6.1. Values are means (n=2 to 3) expressed as a percentage of maximum gape, which was established for each individual, and illustrate the typical pattern observed in most scallops. The characteristic adduction activity of scallops, exhibited immediately upon emersion, results in a gape of $20.5 \pm 6.3\%$ within the first 30 min. of exposure. It was observed that, in the very few animals which did not exhibit adduction, the shell valves remained tightly closed for an extended period, occasionally several hours. However, in typical scallops, gape continues to increase rapidly, reaching $84.3 \pm 17.8\%$ after 6 hours, and maximum gape after approximately 10 hours of emersion, $99 \pm 1.4\%$. Slight changes in shell gape within individual scallops make it difficult to obtain a more precise estimate of the mean time to maximum gape, although observations suggest that scallops exposed to air for longer than 12-15 hours (at 10°C) are not able to reduce gape following reimmersion and subsequently die.

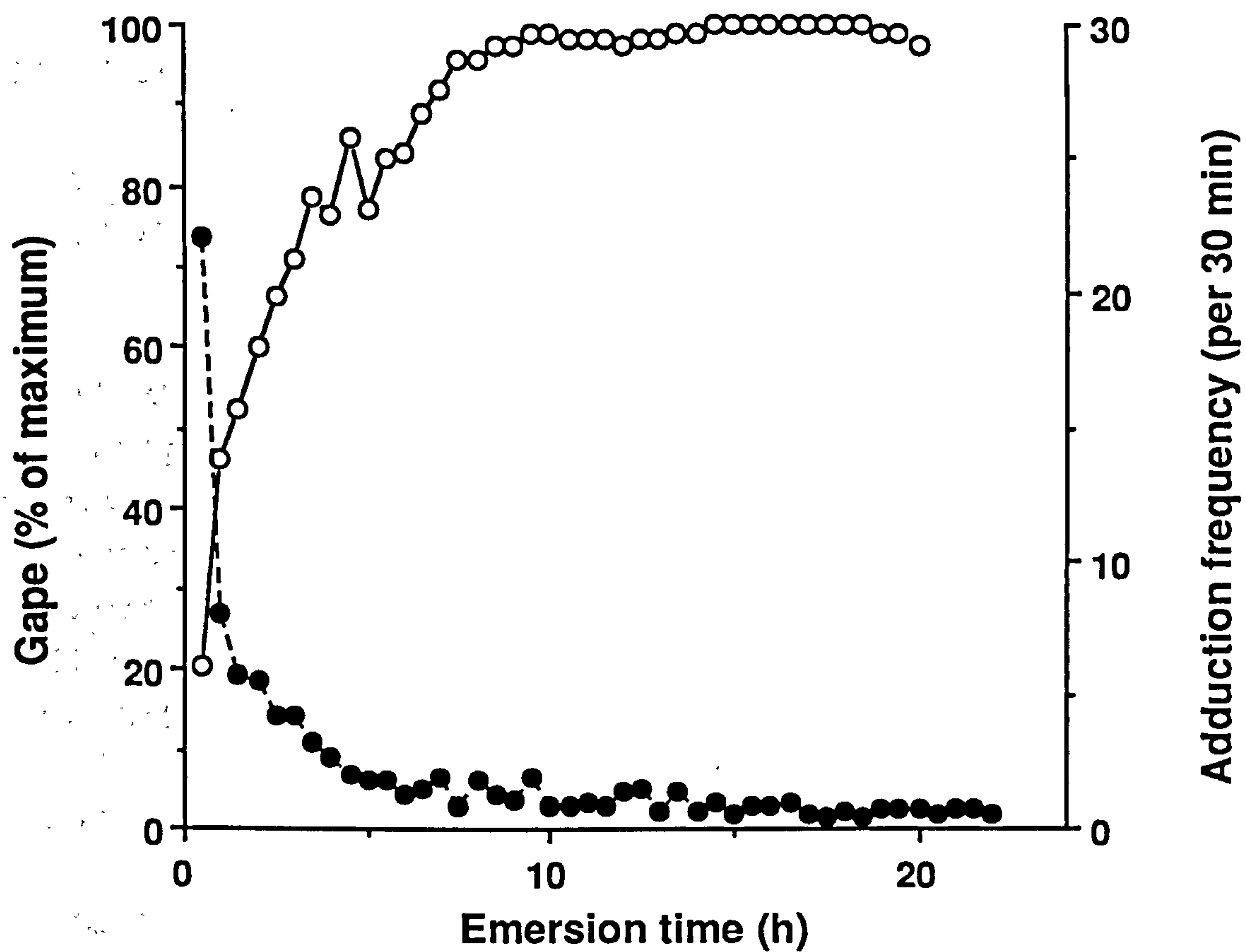
6.2. Histochemistry

6.2.1. Preparation and cryo-sectioning of adductor muscle tissue.

Materials and method

Four scallops were used in these experiments, 2 retained in aerated sea water and 2 emersed in air for 12 hours. The exposed scallops adducted during emersion and exhibited permanent gaping when undisturbed. After this time the adductor muscles from each experimental animal were prepared for cryo-sectioning and staining by plunging the whole scallop into liquid nitrogen until freezing was complete. To avoid the problem of incomplete or delayed freezing each scallop had a wooden wedge forced between the shell valves which prevented closure and

Fig. 6.1 *Pecten maximus*. Changes in valve gape and valve adduction frequency during emersion at $10 \pm 1^\circ\text{C}$ and $95 \pm 2\%$ r.h. Open symbols represent the mean valve gape of 3 individuals expressed as a percentage of their maximum gape. Closed symbols represent the mean adduction frequency per 30 min. of the emersion period ($n=7$). Error bars have been omitted for clarity, see text for values.



hence allowed complete penetration of the liquid nitrogen (-196°C). Upon removal, the shell and viscera were detached from the adductor muscle which, while still frozen, was trimmed and mounted on an aluminium cutting plate using O.C.T. mounting medium (Tissue Tek compound 4583, Miles Scientific).

The tissue blocks were mounted in a freezing microtome (Bright starlet 2212) and, following equilibration to -20°C, sections of phasic and tonic adductor were cut at 20µm. The sections were then mounted on 2cm² glass coverslips which had previously been treated with aminoalkylsilane (Henderson, 1989). Tissue sections were dried at room temperature before staining. All staining procedures were qualitative assays for specific biochemical compounds and, as such, indicate only the relative concentration or activity within different tissue types or under different conditions.

6.2.2. Succinate Dehydrogenase activity (SDH)

Materials and method

Succinate Dehydrogenase activity is indicative of the oxidative capacity of the tissue (Lojda *et al.*, 1976), the enzyme being bound to the inner membranes of the mitochondrial cristae.

Stock solutions of 1M sodium succinate (pH 7.5) and 0.1 M sodium phosphate (Na₂HPO₄, 0.71g/50ml distilled water) with 1mg/ml nitroblue tetrazolium (Sigma N6876)(pH 7.5) were mixed in a proportion of 1:9 to produce a 1ml volume. This solution is a yellow colour and must be discarded if it becomes clear. One drop of this operating solution was then placed on to each tissue section, which were maintained in a covered petri dish on damp filter paper. The sections were incubated at 38°C until a noticeable brown colour developed in the tissue sections. The fluid was then poured off and the sections dehydrated through an alcohol series, cleared in Histoclear (National Diagnostics, New Jersey, USA) and mounted on slides with Histomount.

Results

Succinate Dehydrogenase activity was low in control sections of both types of muscle tissue, since the time taken to produce any noticeable colour was considerable. However, once developed, staining intensity appeared to be slightly greater in the phasic adductor (Plate 6.1), indicating a relatively higher enzyme concentration. The results indicate, however, that both scallop adductor muscles have a relatively low oxidative capacity due to the low numbers of mitochondria. Since this assay reflects the potential for aerobic metabolism, and activity would not diminish this capacity, it was expected that no staining differences would be evident

Plate 6.1

Pecten maximus. Adductor muscle tissue following histochemical staining to reveal Succinate Dehydrogenase activity. Section shows phasic (P), tonic (T) and intermediate (I) muscle fibre types, (x15 magnification).

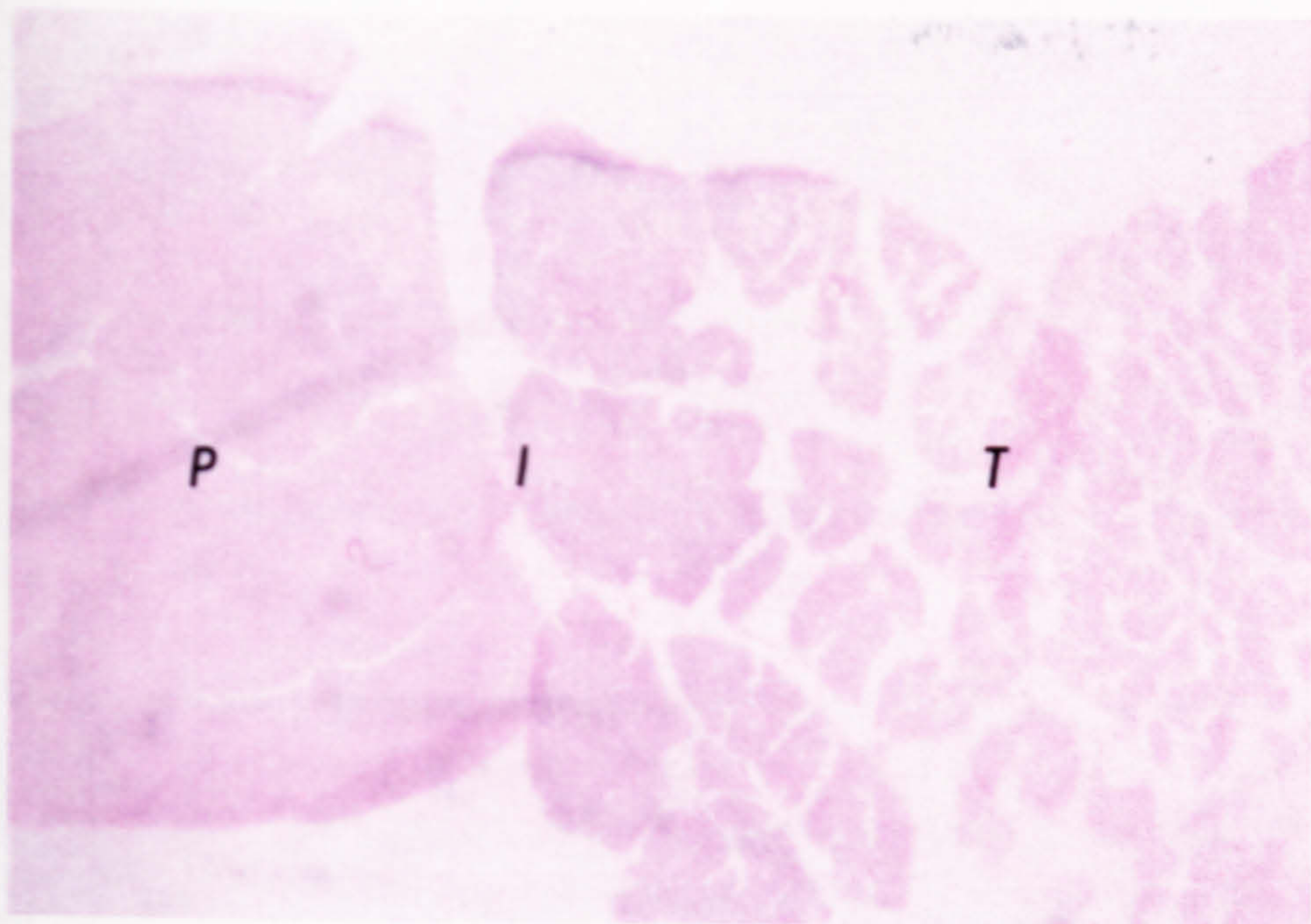
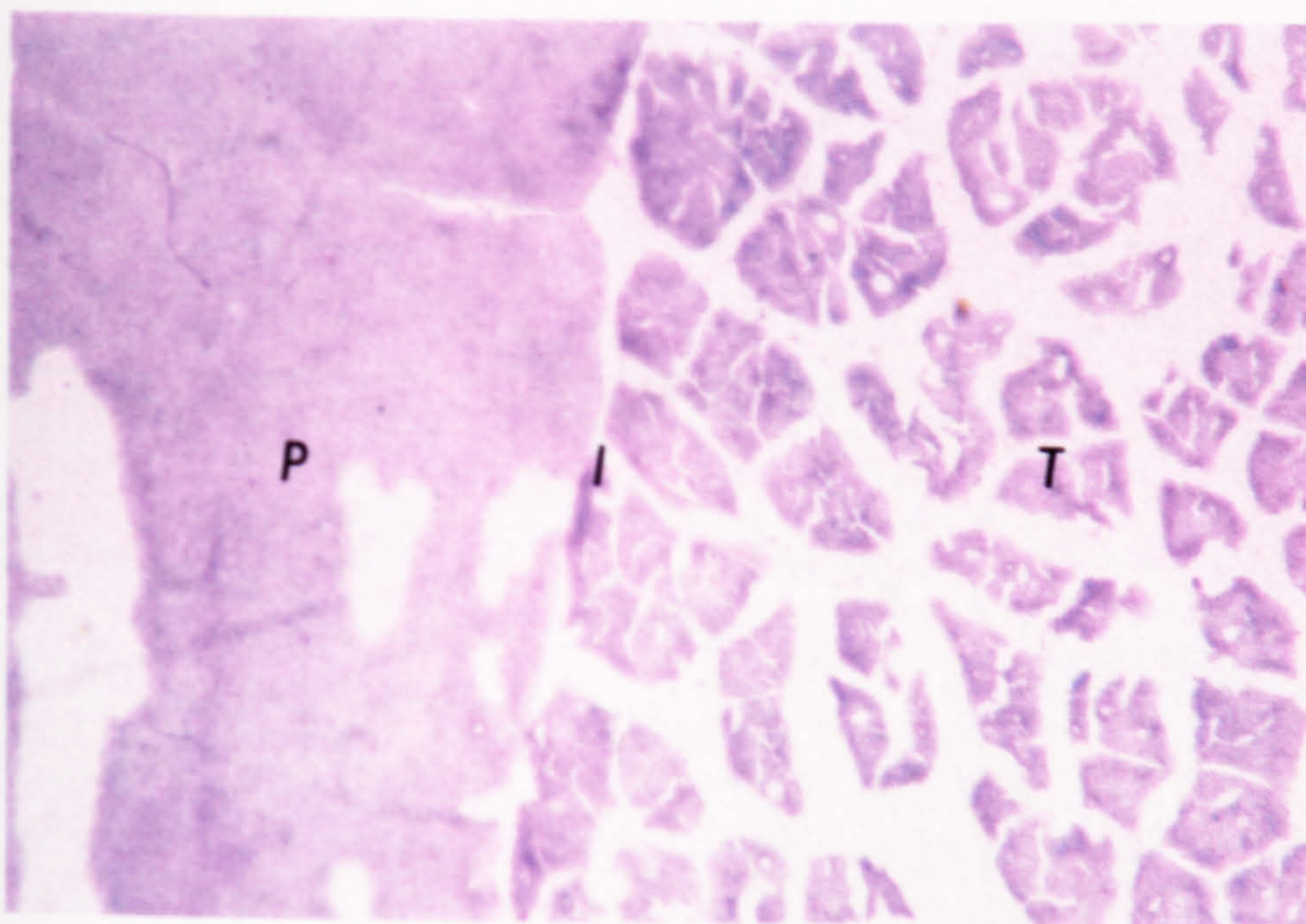


Plate 6.2

Pecten maximus. Adductor muscle tissue following histochemical staining to reveal NADH. Dehydrogenase activity. Section shows phasic (P), tonic (T) and intermediate (I) muscle fibre types, (x15 magnification).



between emersed and control muscle tissues. Staining indicated this to be the case and therefore emersed muscle tissue is not shown.

6.2.3. NADH. Dehydrogenase activity

Materials and method

The capacity of muscle tissue to reoxidise NADH, via the enzyme NADH. Dehydrogenase, is used as a general test for oxidative capacity, since this reaction occurs via the electron-transfer chain located in the mitochondria. It is therefore an indicator of tissue mitochondrial numbers. NADH. Dehydrogenase activity was determined according to the method described by Ogonowski & Lang (1979)

Two ml of 0.1 M sodium phosphate (Na_2HPO_4 , 0.71g/50ml distilled water) with 1mg/ml nitroblue tetrazolium (Sigma N6876)(pH 7.5) were added to a pre-packed tube containing 2 mg of β -NADH (disodium salt) (Sigma chemicals no. 340-102) (1.28mM). One drop of the resultant solution was placed onto the tissue which was then incubated in a petri dish (as in 6.2.2) at 38°C until a grey/blue colour developed in the tissue section. The drop of solution was then poured off and absorbed by the filter paper. Sections were then dehydrated through graded alcohols and mounted as described previously.

Results

Staining indicated that NADH was oxidised in control sections of both adductor types (Plate 6.2), although no differences were apparent between the muscle types nor between muscle tissue from emersed (not shown, see 6.2.2) and control scallops. In common with the SDH assay, staining intensity, reflecting the mitochondrial count, was low. This assay confirmed the earlier conclusion that the overall oxidative capacities of the muscles were low, and that adduction activity during emersion, under conditions of reduced oxygen uptake, is likely to be based on anaerobic metabolism.

6.2.4. Periodic Acid Schiffs (PAS) stain for glycogen.

Materials and method

Periodic acid breaks down the 5-carbon rings in glycogen to form aldehydes, which can be stained using Schiffs reagent to produce a magenta colour. Prepared tissue, mounted on cover slips, was first washed in tap water then with distilled water, before soaking in 1% Periodic acid for 20 minutes. Sections were again well rinsed in tap water, then distilled water, prior to a 20 minute treatment with Schiffs reagent. This procedure was carried out in the dark and the reagent discarded after use. The sections were then rinsed with tap water to enhance the stain, dehydrated through graded alcohols (30%-absolute) and HistoClear, before mounting on slides with Histomount. Alternatively, if the sections were not dehydrated in alcohols, an initial fixing with 5% formalin (10 min.) was required.

Results

Stained muscle tissue from control scallops indicated the presence of significant quantities of glycogen in both phasic and tonic (Plate 6.3) muscle types. Stronger colour development in the phasic portion suggested a relatively higher concentration of glycogen in the muscle responsible for rapid valve movements. Glycogen also appears to be stored differently within the two muscle types. Phasic muscle glycogen showed a diffuse distribution throughout the tissue, whereas tonic muscle glycogen appeared to be sited only within the membranes surrounding the individual muscle fibres (Plate 6.4).

PAS staining of muscle tissue from emersed scallops, which had undergone prolonged valve adduction and subsequent gape, revealed that glycogen stores had been utilised (Plate 6.5), as indicated by a reduction in stain intensity when compared with the control section (Plate 6.3). The decrease in glycogen content appeared to be relatively greater in the more active phasic muscle, although neither type showed complete glycogen depletion.

6.3. Determination of adductor muscle glycogen utilization during emersion in air.

Materials and method

The concentrations of glycogen in scallop adductor muscle tissue were determined quantitatively using the method of Keppler and Decker (1974). In this method the 1-4 & 1-6 glycosidic bonds of glycogen are hydrolysed by the enzyme, 1-4, 1-6 amyloglucosidase. Addition of hexokinase catalyses the phosphorylation of glucose by ATP, whilst glucose-6-

Plates 6.3 & 6.4 *Pecten maximus*. Adductor muscle tissue from an immersed scallop following Periodic acid schiffs (PAS) staining for glycogen. Staining intensity reflects glycogen concentration. Sections show phasic (P), tonic (T) and intermediate (I) muscle fibre types. Upper section taken at x60 magnification. Lower section, at x550 magnification, illustrates the localised distribution of glycogen around tonic muscle fibres and the diffuse distribution in phasic muscle.

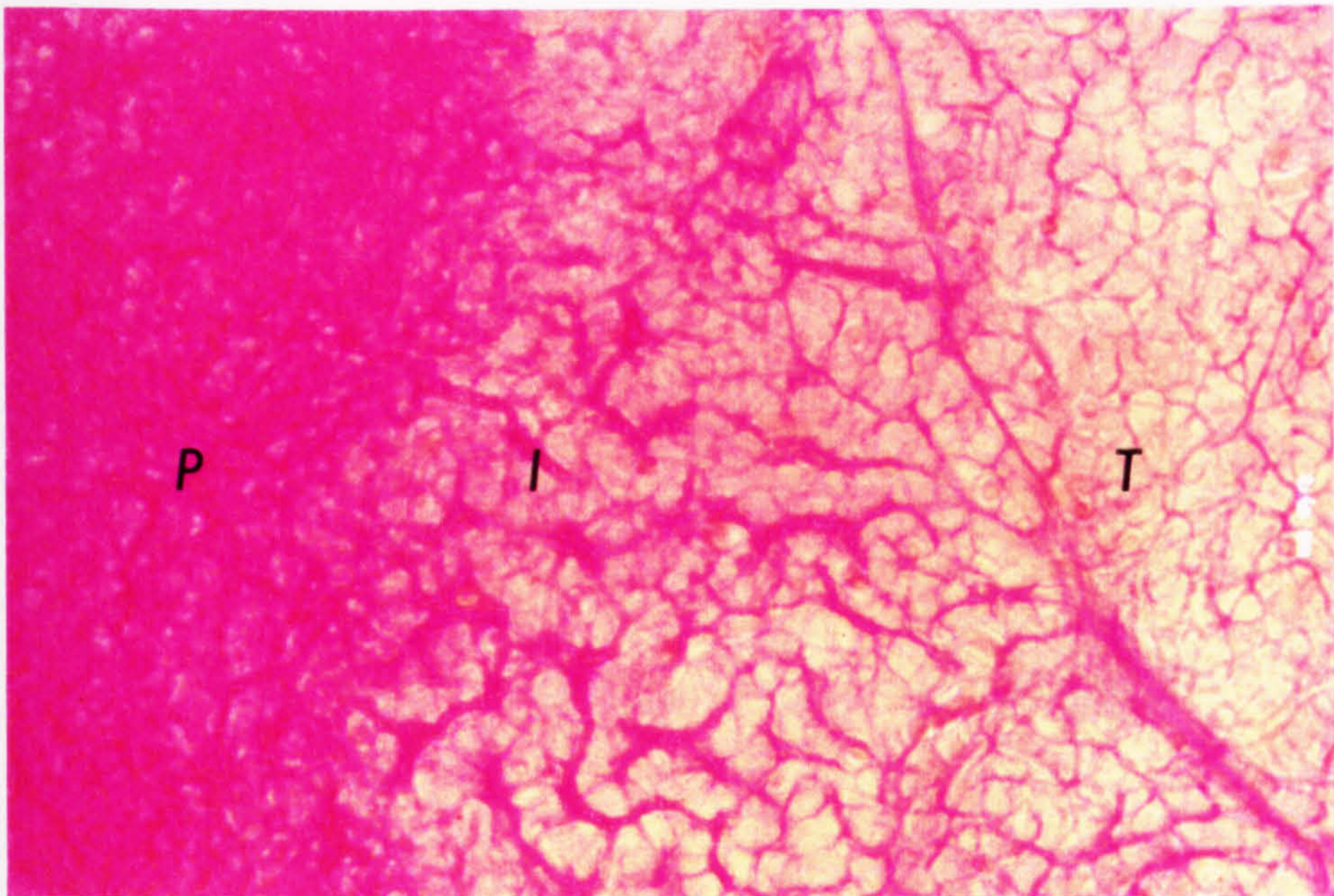
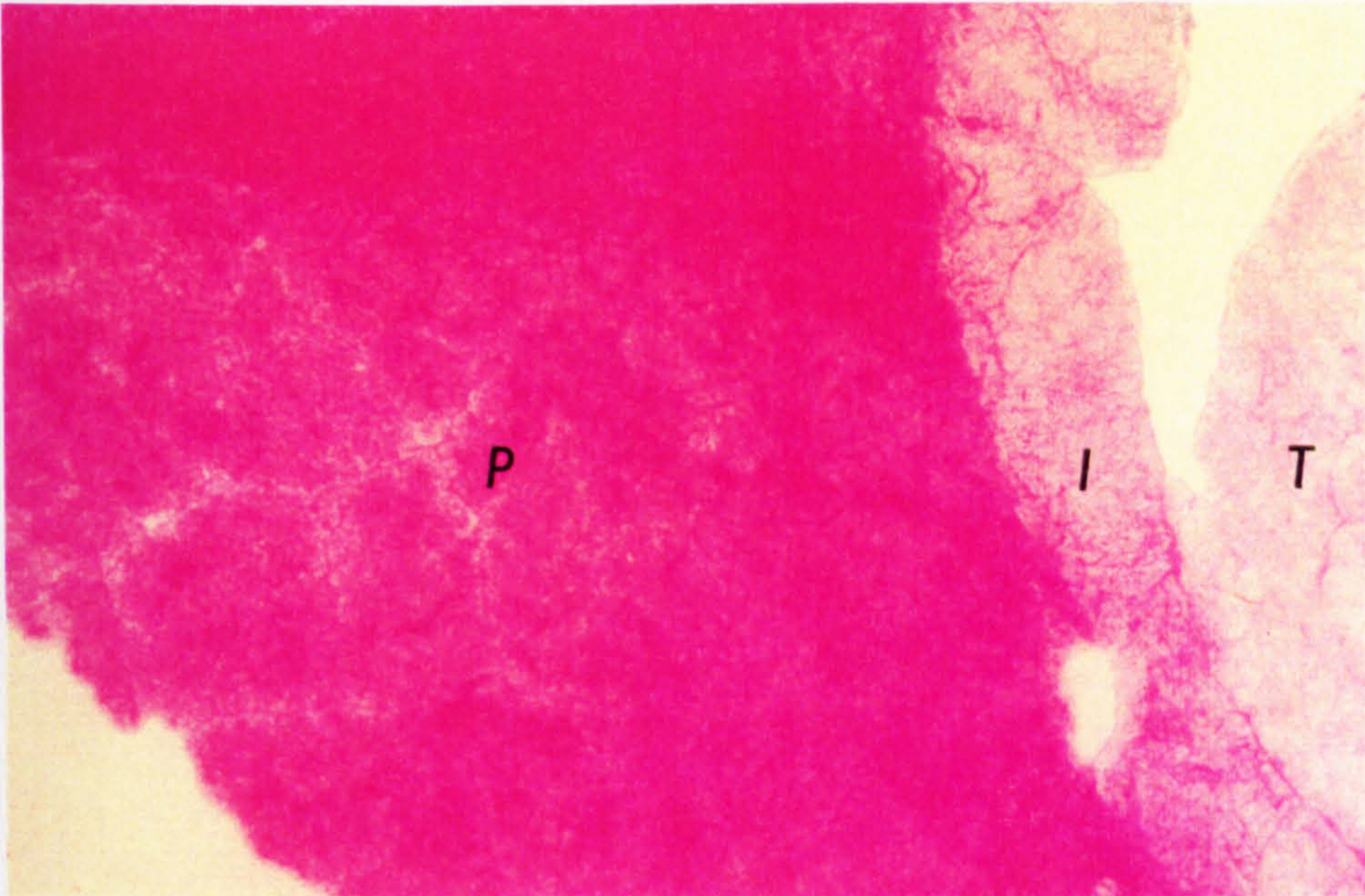
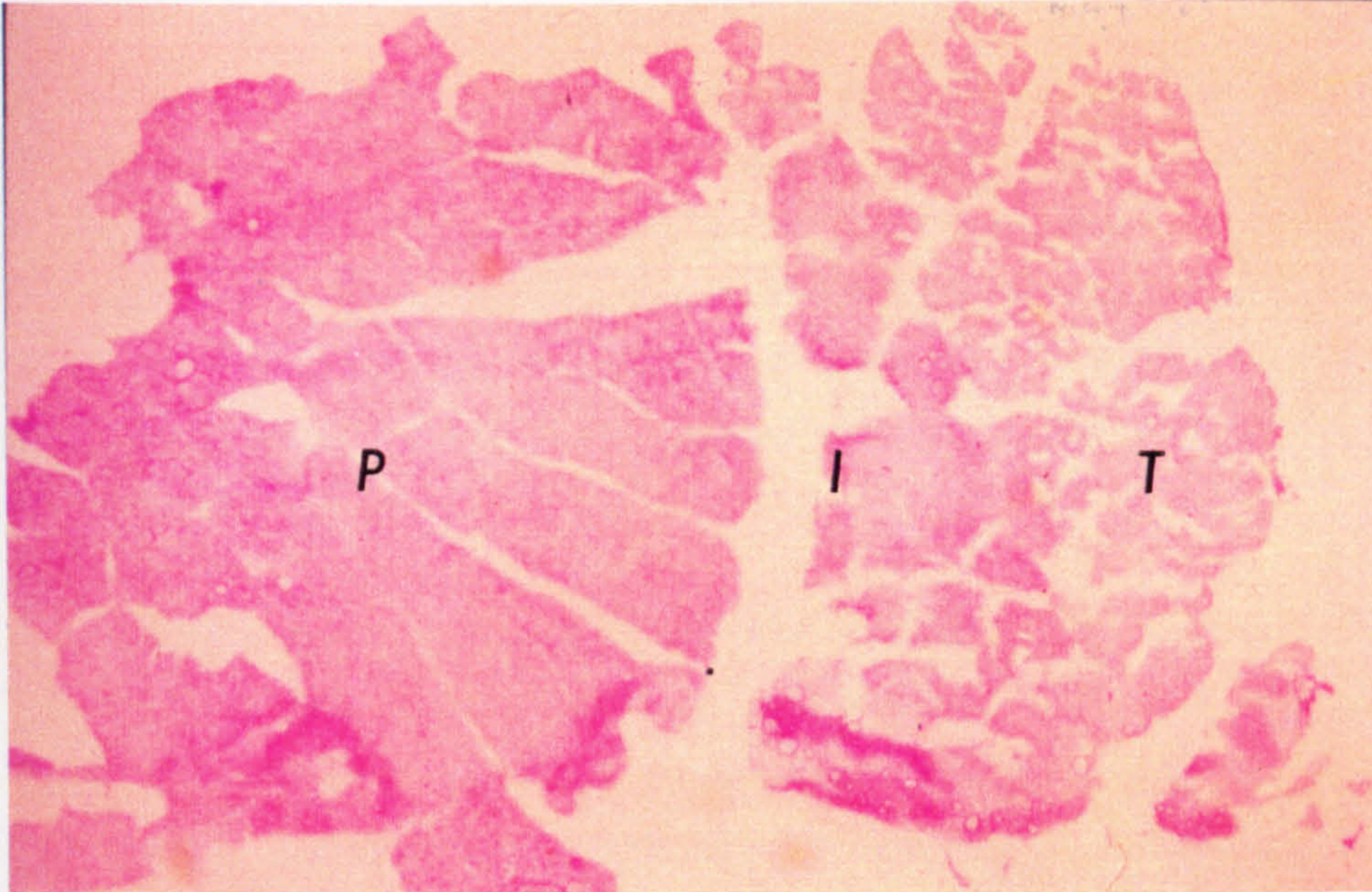


Plate 6.5

Pecten maximus. Adductor muscle tissue from an emerged scallop following Periodic acid schiffs (PAS) staining for glycogen. Scallop emerged at 10°C and 95% r.h. for 12 hours. Staining intensity reflects glycogen concentration which has clearly decreased in comparison to the control tissue (Plate 6.3). Section shows phasic (P), tonic (T) and intermediate (I) muscle fibre types, (x10 magnification).



phosphate dehydrogenase catalyses the oxidation of glucose-6-phosphate to 6-phosphogluconate. The fluorescence of NADPH, produced as a result of these reactions, is then assayed using a spectrofluorophotometer.

- 1) Glycogen or $(\alpha\text{-glucosyl})_n + \text{H}_2\text{O} \Rightarrow (\alpha\text{-glucosyl})_{n-1} + \text{D-glucose}$
- 2) $\text{D-glucose} + \text{ATP} \Rightarrow \text{Glucose-6-phosphate} + \text{ADP}$
- 3) $\text{Glucose-6-phosphate} + \text{NADP}^+ \Rightarrow \text{6-phosphogluconate} + \text{NADPH} + \text{H}^+$

Three groups of 10 scallops were emersed for periods of 12, 24 and 48 hours at a temperature of $10 \pm 1^\circ\text{C}$ and a relative humidity of $95 \pm 5\%$. A control group of 10 scallops were retained in the re-circulating sea-water system at the same temperature. Following the experimental treatments, the adductor muscles were dissected within 10 s and frozen in liquid nitrogen. The tissue was freeze dried (Edwards Modulyo freeze dryer) until a constant weight was obtained, separated into phasic and tonic portions, ground with a mortar and pestle and stored in a desiccator until required.

A 50 mg sample of each powdered muscle type was transferred to an Eppendorf vial to which was added $400\ \mu\text{l}$ of 30% KOH. The vials were then placed in a boiling water bath for 20 minutes. Following the addition of 1 ml of absolute alcohol, the sample was left on ice for 2 hours for the glycogen to precipitate out. Each sample was then centrifuged for 10 minutes at 10,000 r.p.m. and the resultant pellet washed in absolute alcohol and frozen at -20°C . The pellet was then mixed with 1 ml of acetate buffer (2ml 96% acetic acid and 4.87g sodium acetate (35mM) made up to 100ml with distilled water and the pH corrected to 4.8 with NaOH) and $10\ \mu\text{l}$ of amyloglucosidase (Boehringer Mannheim 102849, used without dilution) and incubated at 37°C for 2 hours. From this mixture, $100\ \mu\text{l}$ was removed and added to $700\ \mu\text{l}$ of tris buffer (2.42g Tris and 0.24g magnesium sulphate, 100mM and 10mM respectively, dissolved in 200ml of distilled water and the pH corrected to 7.4 with HCl), and $100\ \mu\text{l}$ each of ATP (Sigma chemicals A-3377, $12.1\text{mg}\cdot\text{ml}^{-1}$) and NADP (Sigma chemicals N-0505, $16.8\text{mg}\cdot\text{ml}^{-1}$). The fluorescence of each sample was then measured using a Shimadzu RF5000 spectrofluorophotometer to provide a background value for NADPH. Excitation and emission wavelengths of the spectrofluorophotometer were set at 340nm and 457nm respectively. After this initial measurement, $10\ \mu\text{l}$ each of hexokinase (Sigma H5500, 1:25 dilution) and glucose-6-phosphate (Boehringer Mannheim 127035, 1:25 dilution) were added to the mixture which was thoroughly shaken and left for 5 minutes at room temperature. The fluorescence of NADPH in each sample was measured again, the change from the initial value being proportional to the concentration of glycogen in each sample. A calibration curve was constructed using a range of glucose standards in order to relate sample fluorescence to glycogen concentration, which could be expressed in glucosyl units.

Results

The glycogen concentrations of phasic and tonic adductor muscle from control animals were $812.3 \pm 289.6 \mu\text{mol. glucosyl units g}^{-1}$ (dry weight) and $504.5 \pm 34.1 \mu\text{mol. glucosyl units g}^{-1}$ (dry weight) respectively ($n=10$ in each case, unless otherwise indicated) (Fig. 6.2). Emersion resulted in a glycogen concentration decrease in both muscle types. After 12 hours exposure the mean glycogen concentration of the phasic adductor was $471.0 \pm 350.0 \mu\text{mol. glucosyl units g}^{-1}$ (dry weight), and tonic adductor was $406.7 \pm 87.9 \mu\text{mol. glucosyl units g}^{-1}$ (dry weight). After 48 hours the concentration of glycogen in the phasic adductor was $118.2 \pm 165.9 \mu\text{mol. glucosyl units g}^{-1}$ (dry weight) ($n=9$) and the tonic adductor was $174.6 \pm 140.1 \mu\text{mol. glucosyl units g}^{-1}$ (dry weight). The glycogen concentration changes in both muscle types over the experimental emersion were statistically significant (ANOVA, $P < 0.01$).

6.4. High Performance Liquid Chromatography (HPLC) analysis of muscle acids

Materials and method

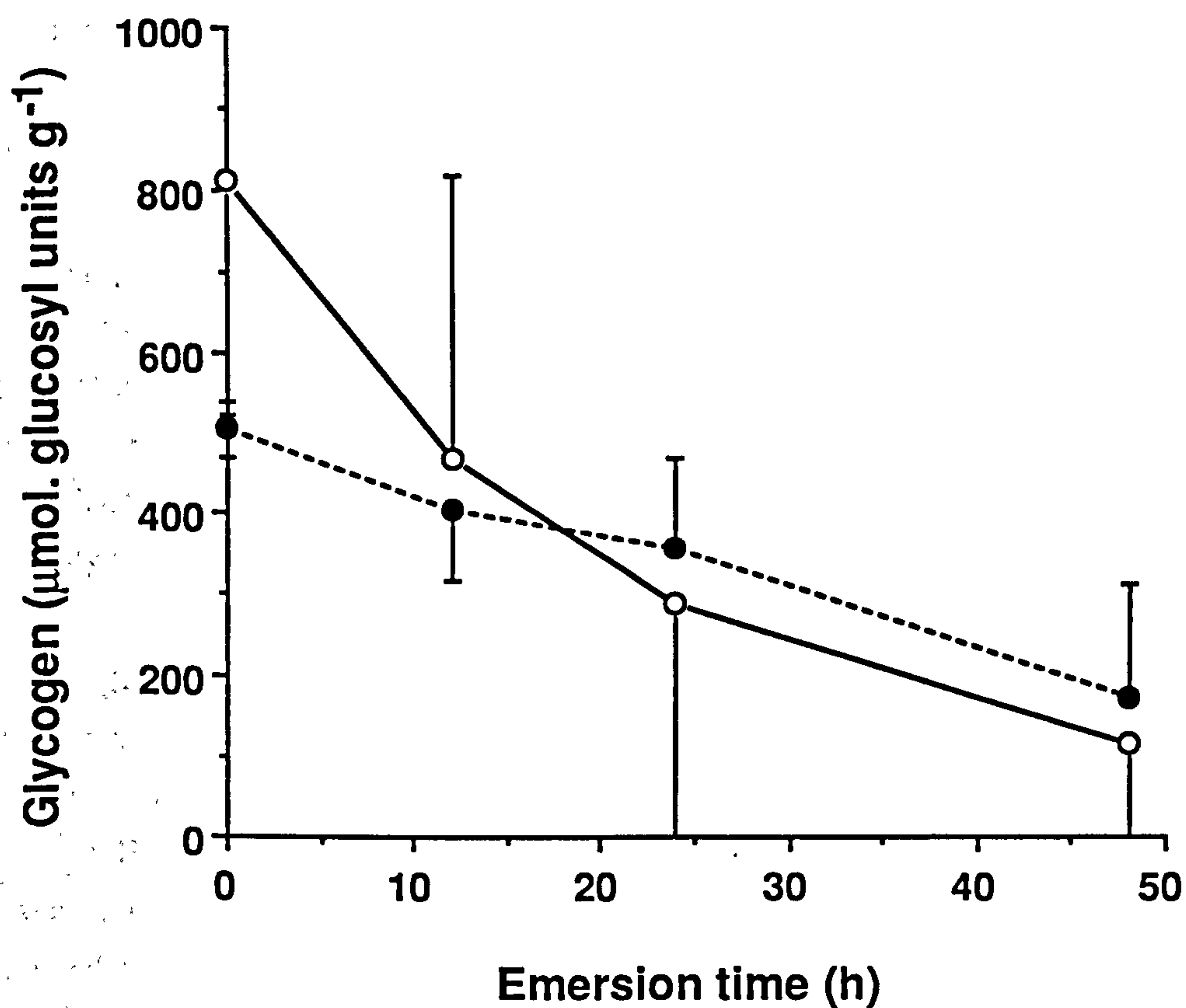
Experimental method and tissue extraction procedure

Four groups of 5 scallops were emersed in fibre-glass tanks (see section 3.2) for periods of 12, 24, 48, and 72 hours at a temperature of $10 \pm 1^\circ\text{C}$ and $95 \pm 5\%$ r.h. A control group of 5 animals were retained in sea water. Following emersion, all scallops were quickly dissected and the adductor muscles removed. Muscles were frozen, dried and powdered according to the method described in section 6.3.

The tissue extraction method was based on the procedure described by Gäde *et al.* (1978). Samples of powdered tissue (50 mg) were weighed into Eppendorf vials into which 500 μl of chilled perchloric acid (PCA) (0.3M) was added. After mixing thoroughly, samples were centrifuged at 10,000 r.p.m. for 10 minutes and the supernatants removed and stored on ice. A further 500 μl of chilled PCA (0.3M) was added to each remaining pellet, mixed again, and centrifuged at 10,000 r.p.m. for 20 minutes. The resulting supernatants were added to the original supernatants and the mixtures neutralized using 2M potassium bicarbonate (KHCO_3). Addition of KHCO_3 resulted in the production of CO_2 gas within the vials and it was necessary to allow this gas to escape before mixing. The volume of KHCO_3 required to neutralize each sample was noted for use in dilution factor calculations. The mixtures were centrifuged for a final time to remove the precipitate of potassium perchlorate and the supernatants separated and stored at -70°C until required.

Fig. 6.2

Pecten maximus. Changes in the glycogen concentration (expressed in glucosyl units) of adductor muscle during emersion at $10 \pm 1^\circ\text{C}$ and $95 \pm 2\%$ r.h. Values are means (\pm S.D.) per gram of dry tissue ($n=10$ at each time point). Open symbols represent phasic (striated) adductor and closed symbols represent tonic (smooth) adductor muscle. Error bars omitted for clarity where appropriate.



HPLC analysis method

The concentrations of organic acids present in the muscle tissue samples were estimated using a Gilson HPLC system. The system consisted of the following units (all Gilson, France); UV detector (116), dynamic mixer (811), manometric module (802c) and 2 solvent pumps (302) controlled by Gilson 715 software. Each extracted muscle sample was passed through a 0.4 μm filter to remove any suspended material and a 20 μl volume was injected onto the column. The column used was a Brownlee Polypore H organic acid analysis column (220x4.6 mm with 10 μm diameter packing) which was eluted with 0.01M H_2SO_4 at a rate of 0.3ml.min.⁻¹. The column was maintained at 70°C in a Jones chromatography column heater to improve peak separation. Organic acids were detected at a wavelength of 210 nm and the results recorded by the software system. Run time was 20 minutes.

The following standards were run over a range of concentrations (between 0.1 & 10mM) and calibration curves constructed to relate peak area to acid concentration; acetate, formate, fumarate, lactate, malate, malonate, oxaloacetate, propionate, pyruvate, succinate.

Results

Examples of typical HPLC organic acid chromatograms for phasic and tonic adductor muscles are shown in Fig. 6.3 a,b. Quantitative data from individual samples, which relate to identified acids, are shown in Table 6.1. The following acids were identified in both muscle types; malate, fumarate, succinate and propionate, with all other standard acids being absent, unidentifiable or occurring irregularly in negligible quantities. Acid concentrations were generally higher in tonic muscle tissue.

A regular pattern of organic acid accumulation was observed in all samples, with the highest concentrations usually recorded after 24 hours. The exception was propionate which showed a high concentration after 12 hours, but the highest concentration after 48 hours of emersion. Both muscle types showed a decrease in all organic acids, except propionate, between 24 and 48 hours. The concentrations of malic, fumaric and succinic acid in phasic muscle then increased between 48 and 72 hours, whereas acid concentrations in tonic muscle decreased. Propionic acid was not detected in any 72 hour muscle sample.

The high variation in acid concentration within each group, coupled with the relatively small sample sizes, made statistical analysis rather inconclusive, although in a number of cases the changes between 0-24 and 24-48 hours were statistically different (ANOVA, $P < 0.05$).

Fig. 6.3a

Pecten maximus. HPLC chromatograms showing metabolic acid concentrations in phasic adductor muscle after 12 (upper trace) and 24 (lower trace) hours emersion at 10°C and 95% r.h. Identifiable peaks correspond to the solvent front (SF), malate (M), succinate (S), fumarate (F), and propionate (P). Vertical axis is scaled to the highest peak and all others are shown as a percentage.

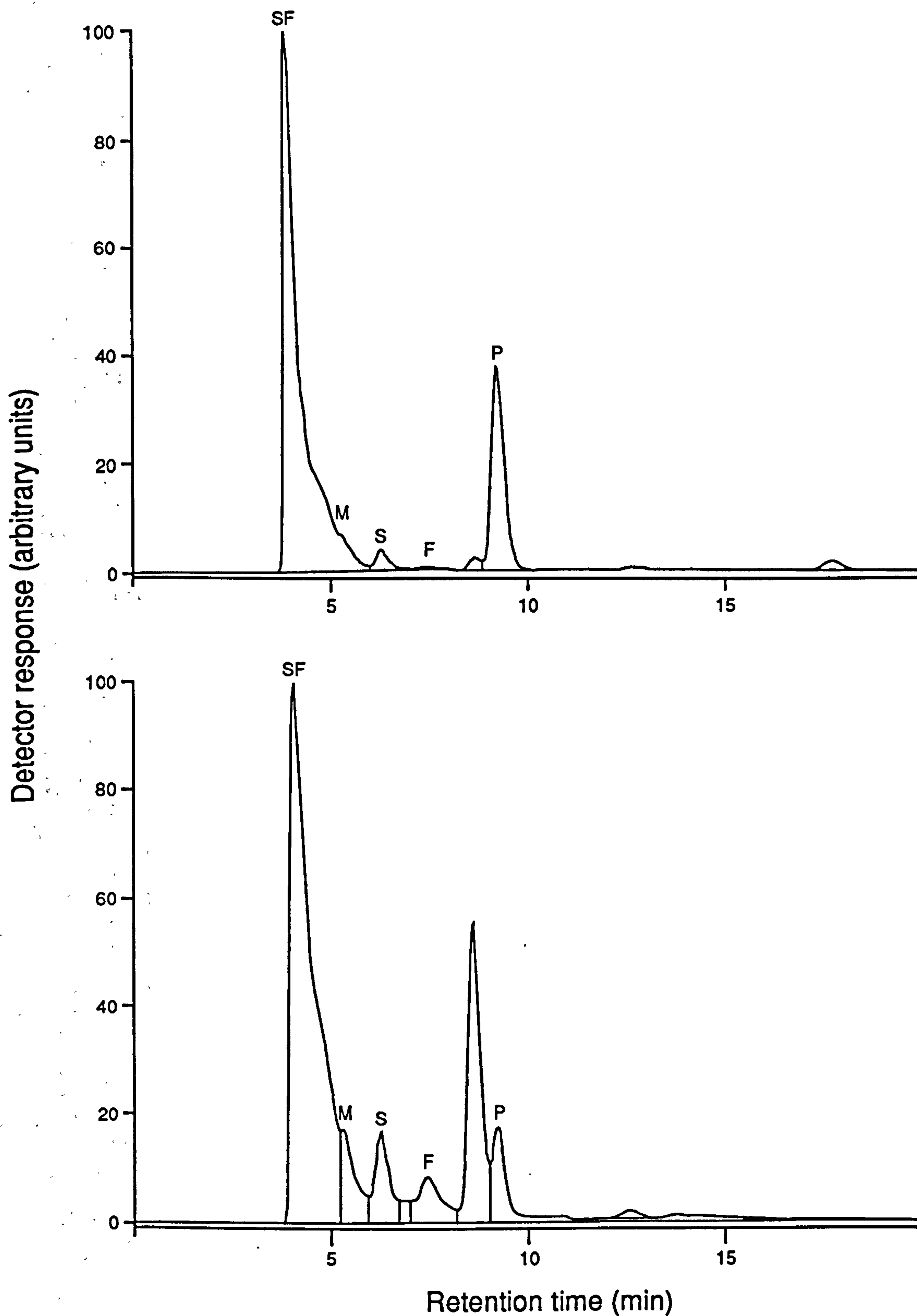


Fig. 6.3b

Pecten maximus. HPLC chromatograms showing metabolic acid concentrations in tonic adductor muscle after 24 (upper trace) and 48 (lower trace) hours emersion at 10°C and 95% r.h. Identifiable peaks correspond to the solvent front (SF), malate (M), succinate (S), fumarate (F), and propionate (P). Vertical axis is scaled to the highest peak and all others are shown as a percentage.

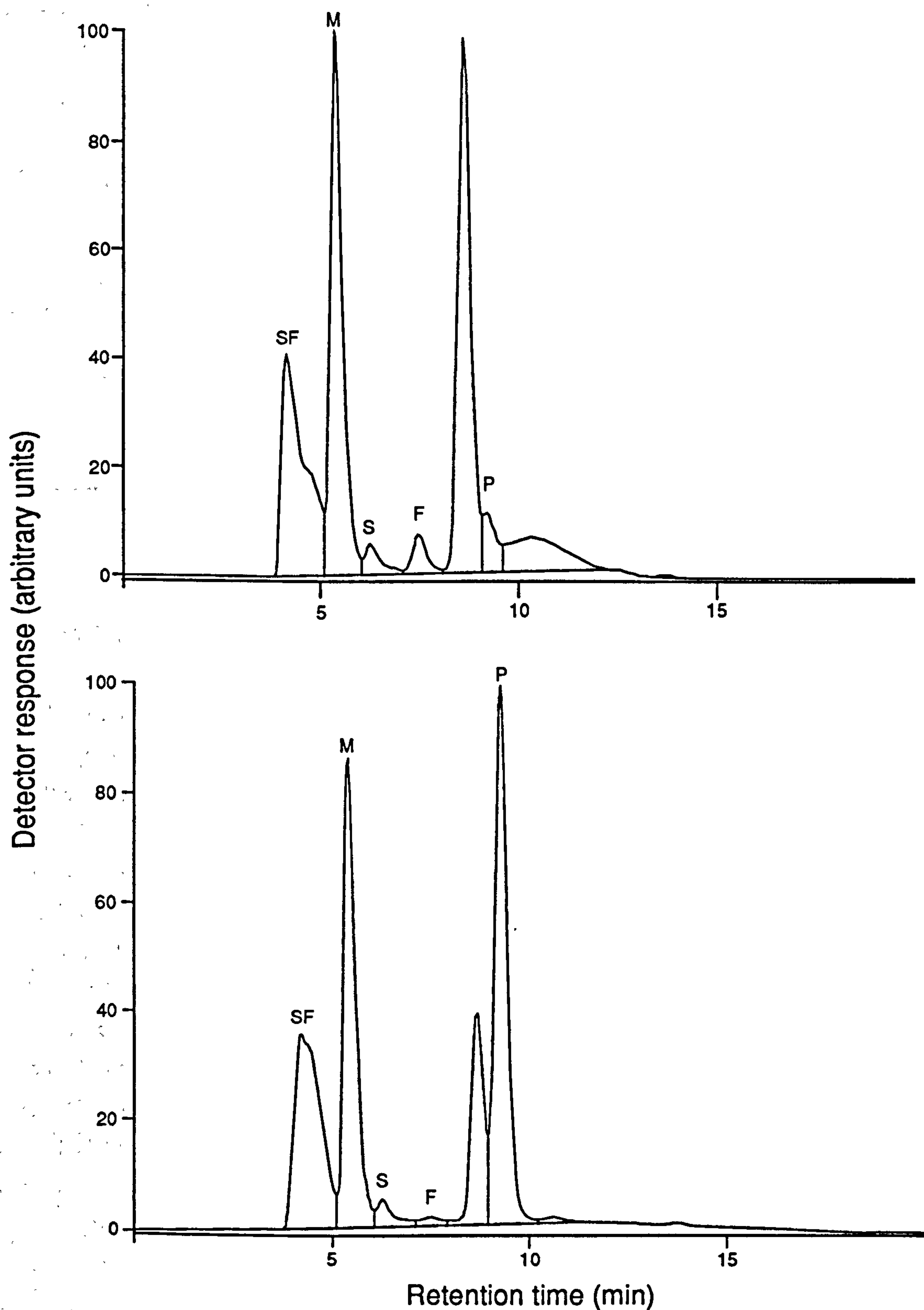


Table 6.1 Changes in organic acid concentrations of *Pecten maximus* adductor muscle during aerial emersion at 10°C and 95% r.h. Data are means (+1S.D. except where n=1), n=1 to 4. Symbol * denotes absence of acid from sample.

Muscle type	Emersion time (h)	Organic acid $\mu\text{mol. g}^{-1}$ (dry weight)			
		Malate	Fumarate	Succinate	Propionate
Phasic	0	52.35	0.02 (.02)	50.3 (4.8)	165.3 (47.1)
	12	*	0.02 (.01)	30.1 (4.7)	442.1 (327.7)
	24	72.7	0.19 (.11)	68.9 (3.6)	87.3 (26.7)
	48	40.7 (4.1)	0.03 (.01)	33.3 (9.8)	829.5 (540.9)
	72	63.8 (17.9)	0.24 (.25)	47.2 (16.4)	*
Tonic	0	597.8 (6.1)	0.11 (.04)	58.3 (6.4)	177.6
	12	626.0 (150.0)	0.07 (.02)	43.7 (5.9)	591.7 (426.1)
	24	734.3 (88.8)	0.17 (.14)	104.6 (60.2)	164.5 (30.8)
	48	667.5 (80.4)	0.11 (.07)	50.2 (12.2)	882.2 (623.5)
	72	629.0 (289.0)	0.04 (.02)	48.2 (10.3)	*

6.5. HPLC analysis of muscle adenine nucleotides

The experimental emersion procedure and muscle tissue preparation was carried out according to the method described in 6.4. Five scallops were maintained in aerated sea water during the emersion period and were used to provide control values for muscle nucleotides.

Reference was made to Perret (1987) for the analysis method. Extracted samples were injected into a 20 μl loop connected to an Anachem S5SAX-2560 column (250x4.6mm and 10 μm diameter packing) which was eluted with 2 solvents; A (0.04M KH_2PO_4 , pH 2.8) (Fisons HPLC grade reagent) and B (0.5M KH_2PO_4 + 0.8M KCl, pH 2.7) (BDH, HiPer-Solv). The solvents were made up with HPLC grade water and pH was adjusted with orthophosphoric acid (BDH, HiPer-Solv). The solvents were run as a linear gradient from 100% A to 100% B in 14 min, 100% B 14-16 min, followed by 100% B to 100% A between 16-17 min. The remaining 3 min were run at 100% A, and total run time was 20 min. Flow rate was 1 ml. min⁻¹. Standards of adenosine-5'- triphosphate (ATP), -diphosphate (ADP) and -monophosphate (AMP) (all Sigma sodium salts) were run at concentrations of 5, 2.5, 1.25, 0.5, 0.25 and 0.125 mM to provide a calibration curve relating peak area to nucleotide concentration.

After tissue adenylate concentrations had been determined, the relative changes in each nucleotide were compared by application of an index, known as the Adenylate Energy Charge (AEC) (Atkinson and Walton, 1967). AEC was calculated using the following equation:

$$\text{AEC} = \frac{[\text{ATP}] + 1/2[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}$$

Results

Data from two HPLC analyses were pooled to provide values for total adenine nucleotide concentrations and for calculation of the AEC. Changes in muscle adenine nucleotide concentrations are shown in Table 6.2.

Table 6.2 Changes in adenine nucleotide concentrations of *Pecten maximus* adductor muscle tissue during aerial emersion at 10°C and 95% r.h. Data are pooled means (+1S.D.) from two analyses, n=6 to 8.

Muscle type	Emersion time (h)	Adenine nucleotide concentration μmol. g ⁻¹ (dry weight)		
		AMP	ADP	ATP
Phasic	0	4.0 (2.6)	6.2 (2.9)	48.6 (12.0)
	12	3.5 (1.1)	5.2 (1.7)	43.4 (6.6)
	24	10.6 (5.4)	19.6 (5.3)	20.6 (4.5)
	48	26.1 (12.6)	18.3 (11.9)	14.6 (8.3)
	72	18.3 (13.8)	12.4 (5.2)	26.6 (14.3)
Tonic	0	5.5 (2.1)	8.9 (1.8)	13.7 (3.6)
	12	3.5 (1.1)	9.8 (9.4)	14.4 (4.6)
	24	6.2 (1.3)	11.7 (2.0)	9.6 (1.1)
	48	14.7 (5.0)	10.1 (1.3)	5.8 (1.7)
	72	13.5 (4.3)	12.3 (2.0)	7.4 (4.7)

The concentrations of AMP and ADP in phasic muscle increased significantly during the first 48 hours of emersion (ANOVA, P<0.01). Both concentrations then decreased between 48 and 72 hours. The ATP concentration exhibited a different trend with a significant and sustained decrease up to 48 hours (ANOVA, P<0.01), followed by a marked increase between 48 and 72

hours. Adenine nucleotide concentration changes in the tonic adductor showed a broadly similar pattern during extended emersion, although actual concentrations were lower. Again, AMP increased significantly between 0 and 48 hours (ANOVA, $P < 0.01$), followed by a slight decrease after 72 hours. However, ADP concentration peaked at 24 hours (ANOVA, $P < 0.05$) then decreased slightly, before increasing again up to 72 hours. Tonic adductor ATP concentrations showed similar changes to phasic muscle. The overall changes in muscle nucleotides are shown in Figs. 6.4 a,b (phasic and tonic muscles respectively). Both sets of chromatograms illustrate the change from a predominantly high ATP concentration in muscle before emersion, indicating a normal situation, to a predominantly high AMP/ADP concentration in 72 hour emersed muscle, indicating a sub-optimal condition.

When these changes are expressed in relation to each other, i.e. using AEC ratios (Fig. 6.5), the similarity of the trends can be observed. In both phasic and tonic adductors energy charge did not change during the first 12 hours of emersion. Between 12 and 48 hours energy charge decreased significantly from 0.88 to 0.40 in phasic muscle and from 0.71 to 0.36 in tonic muscle (ANOVA, $P < 0.01$). In both cases the energy charge ratio then increased slightly between 48 and 72 hours, to 0.57 and 0.40 respectively. However, this change was not statistically significant ($P > 0.05$).

Fig. 6.4a *Pecten maximus*. HPLC chromatograms showing adenine nucleotide concentrations in phasic adductor muscle before (upper trace) and after (lower trace) 72 hours emersion at 10°C and 95% r.h. Identifiable peaks correspond to adenosine '5- monophosphate (AMP), - diphosphate (ADP), and triphosphate (ATP).

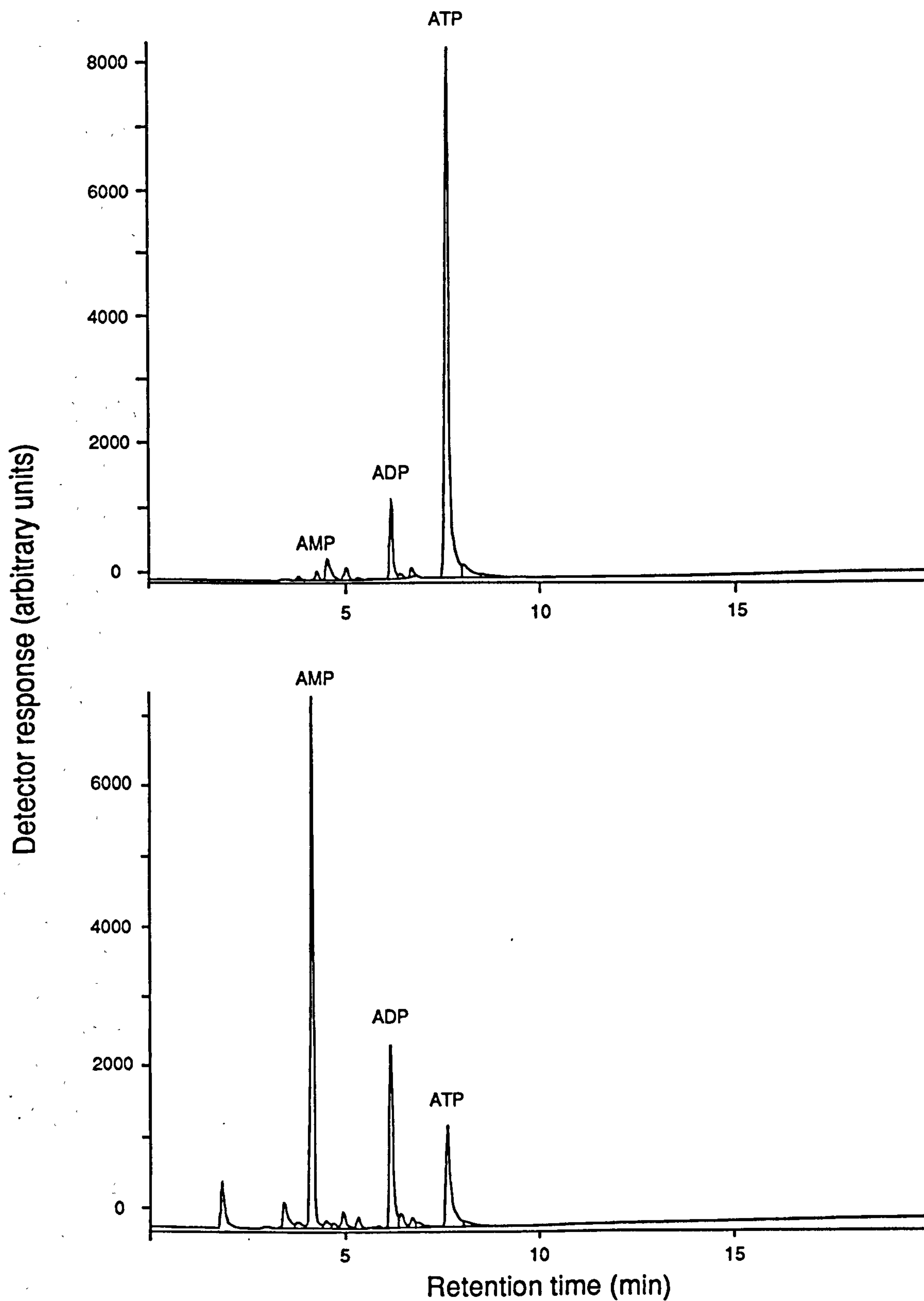


Fig. 6.4b *Pecten maximus*. HPLC chromatograms showing adenine nucleotide concentrations in tonic adductor muscle before (upper trace) and after (lower trace) 72 hours emersion at 10°C and 95% r.h. Identifiable peaks correspond to adenosine 5'-monophosphate (AMP), -diphosphate (ADP) and -triphosphate (ATP).

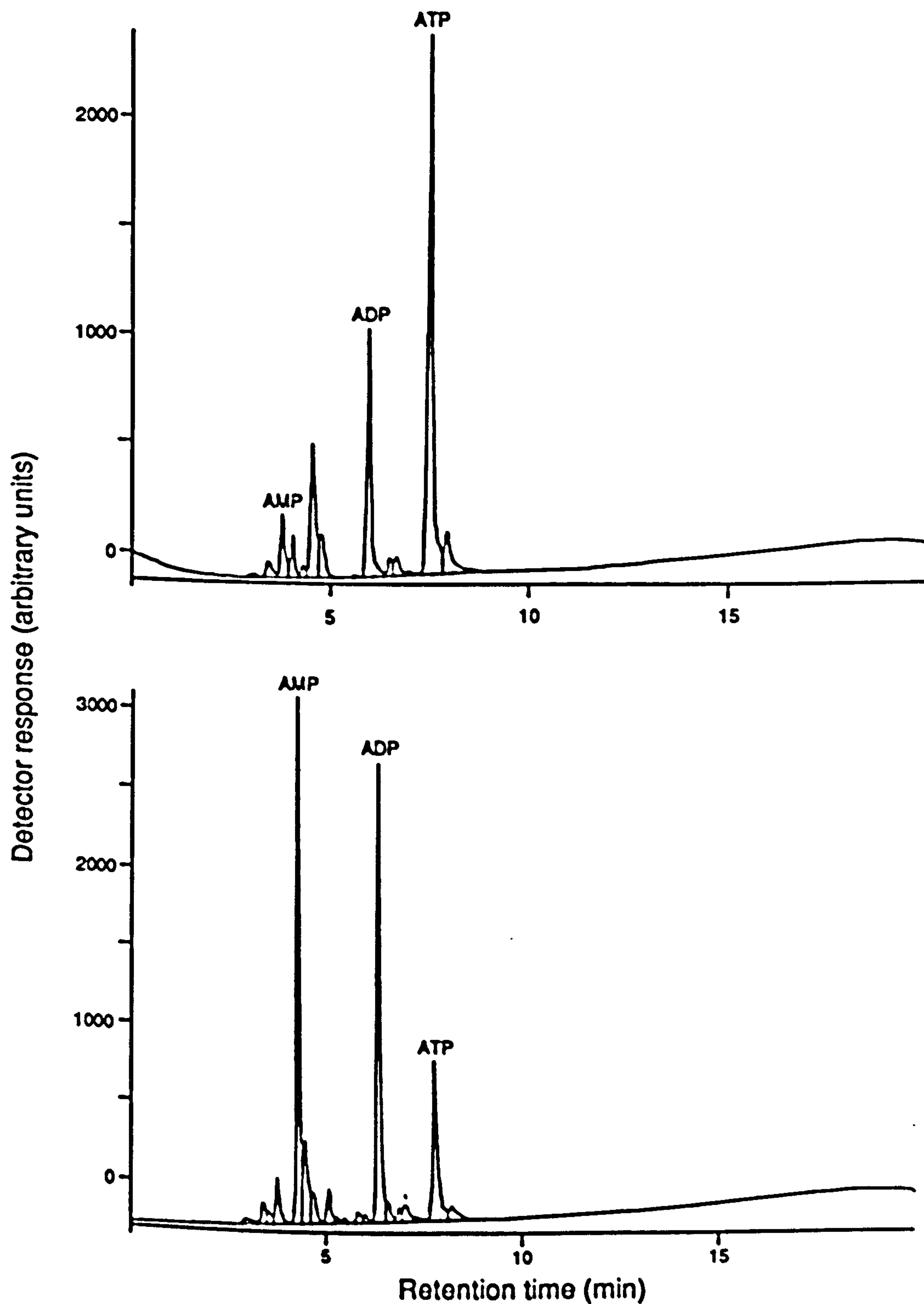
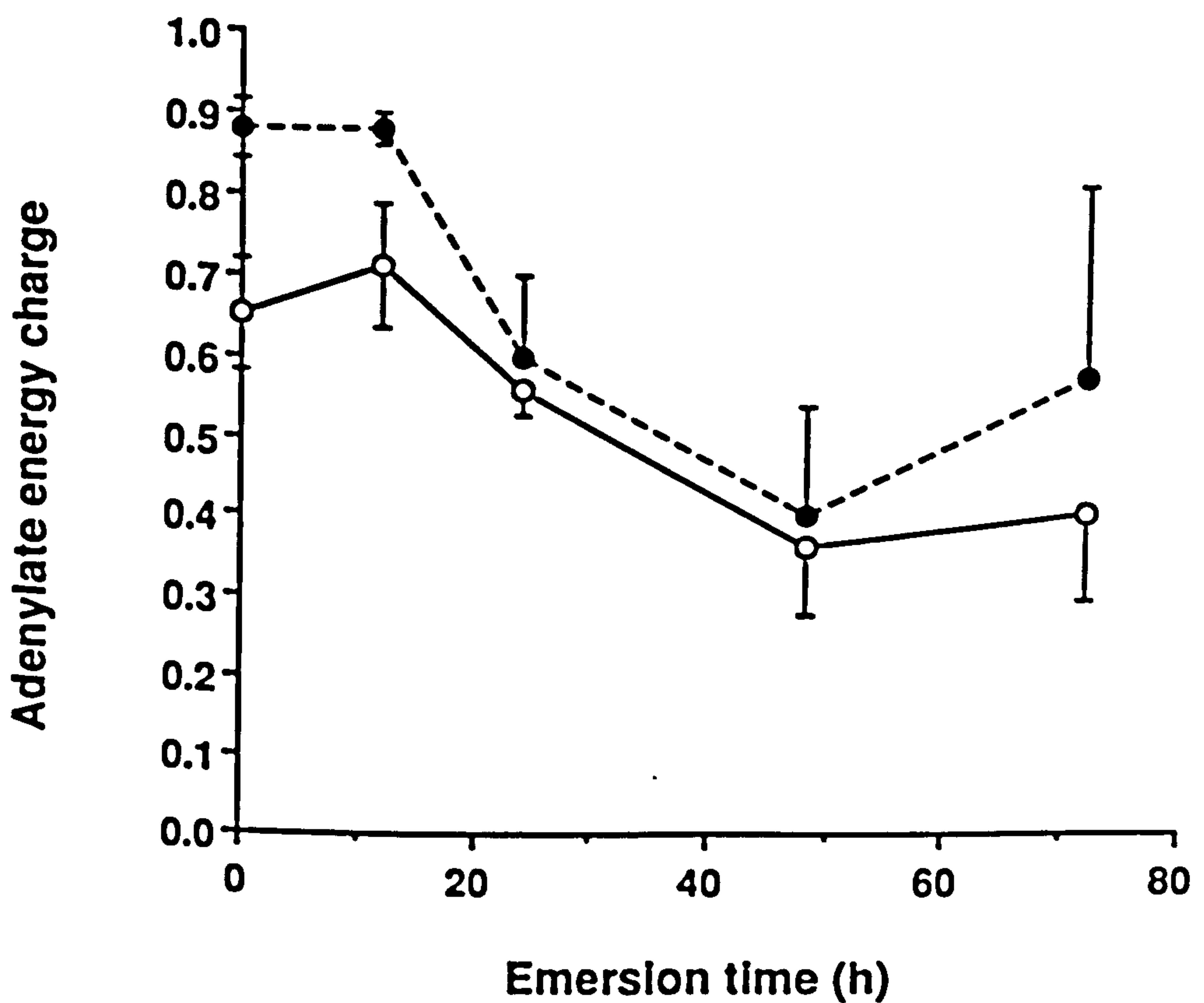


Fig. 6.5 *Pecten maximus*. Changes in the adenylate energy charge of adductor muscle tissue during emersion at $10 \pm 1^\circ\text{C}$ and $95 \pm 2\%$ r.h. Values shown are means (\pm S.D.) ($n=6$ to 8). Closed symbols represent phasic (striated) adductor muscle and open symbols represent tonic (smooth) muscle.



Discussion

The degree of valve gape exhibited by emerged scallops is related to the valve adductions which the animal makes. Since gape increased with emersion time and adduction frequency decreased, it is suggested that gaping is caused by fatigue of the adductor muscles. Closure of the shell valves involves both adductors, phasic for initial closure (using a phasic contraction) and tonic for sustained closure (using a tonic contraction), and permanent gaping therefore implies failure of both muscles. Although generally classified for simplicity as distinct phasic and tonic adductors, the tonic (smooth) muscle is capable of both forms of contraction due to the presence of 2 different muscle fibre types (Rüegg, 1961). This is reflected in the fact that the tonic adductor is also partially involved in valve closure (de Zwaan *et al.*, 1980). In addition, although the tonic adductor is categorised as a 'paramyosin smooth' muscle, the proportion of paramyosin in each fibre type is also different, ranging from 54-57% in the tonic type fibres and 26-30% in the phasic fibres. In comparison, the striated (phasic) adductor, which undergoes only phasic contraction has a paramyosin content of <1% (Rüegg, 1961).

The muscle fibres of the smooth adductor which have a high paramyosin content are also capable of a sustained contraction known as 'catch', which is characterised by maintenance of tension at a very low energy cost (Rüegg, 1971). This mechanism, which has been reported in many bivalves, is therefore an important factor in enabling intertidal species to maintain valve closure during tidal exposure when metabolic rate is reduced (Coleman & Trueman, 1971). The 'catch' state is also exhibited by scallops under certain circumstances, possibly in response to sea star predators (Thomas & Gruffydd, 1971). It seems clear, however, that *Pecten maximus* does not enter an efficient catch state during emersion, perhaps making the animal more likely to gape. A possible explanation for the absence of 'catch' may be derived from investigations into the anterior byssus retractor muscle (ABRM) of *Mytilus galloprovincialis* (Jewell, 1959). This species is also capable of phasic and tonic muscle responses, including a catch state, which is utilised during tidal exposure. Preparations of the isolated muscle, in which the muscle was fixed at one end, but attached to a transducer at the other, showed that both types of contraction could be induced by experimental stimulation. Rapid release of the stimulated muscle, by controlled movement of the transducer apparatus, however, resulted in rapid and complete loss of tension. When this procedure was applied to a phasic contraction full muscle tension was quickly redeveloped. During a tonic contraction, however, muscle tension returned to only 25% of the previous level. If this experimental situation is compared with the behaviour of an emerged scallop, then a rapid valve adduction is analogous to the rapid tension release, hence the catch portion of the tonic adductor is never permitted to develop sufficient tension to hold the valves closed in the most energy efficient way. Scallop muscle activity during emersion is therefore predominantly of a phasic type with a tonic, though not catch, component which

gradually fatigues and releases smooth muscle tension. This results in an increasing gape between successive adductions.

The reason *Pecten maximus* adducts at all during emersion is unclear, since the behaviour is usually associated with a predator-escape response (Thomas & Gruffydd, 1971). However, in a study of the pearl oyster, *Pteria martensii*, in relation to water oxygen content and valve activity, Miyauti (1968) showed that shell movements were unchanged until an oxygen concentration of 1.5 ml.l^{-1} (approximately 44 Torr) was reached. This oxygen concentration, which coincided with the critical oxygen tension (P_c), caused an increase in both adduction frequency and the extent of valve opening. Additional work on the same species found that a water oxygen content of 0.2 ml.l^{-1} (6 Torr) resulted in valve gaping after several short periods of closure (Miyauti & Irie, 1966). Observations made during this study also suggested that hypoxic water (ca. 35 Torr) caused wide valve gaping in *Pecten maximus* within 30 min. It seems probable that valve adduction during emersion also occurs in response to low oxygen tension, caused by the reduced ability of collapsed gill filaments to absorb atmospheric oxygen (see chapter 4). The consequence of repetitive phasic adduction is that energy expenditure, measured as increased oxygen consumption, is about 3 times greater than if the valves were held closed in a state of catch (Withers, 1992). It follows that, compared with energy-conserving bivalves, the scallop would require a relatively greater amount of energy if it is to avoid muscle failure. This would normally require use of the more efficient, energy productive aerobic pathways, but as previously shown (Fig. 4.2), oxygen uptake decreased during emersion suggesting that anaerobic pathways are likely to be employed within a short time of the initial emersion. Since gaping does occur, a number of possible explanations are proposed;

1. Depletion of the glycogen energy store is such that insufficient energy can be produced.
2. The available metabolic pathways are incapable of producing sufficient energy to maintain sustained contraction.
3. The adoption of anaerobic energy pathways results in the production of end products which adversely affect the contractile mechanism of the adductor muscles.

Preliminary histochemical investigations indicated that although the principal energy substrate, glycogen, was used during emersion it was not fully depleted in either muscle type. Additional staining procedures showed that the oxidative or aerobic capacities of both muscle types were relatively low. The results of the SDH and NADH Dehydrogenase activity assays were indicative of a low mitochondrial count, since colour development was neither rapid nor intense. This view is supported by the findings of Mattison & Beechy (1966) who reported small numbers of mitochondria in *Pecten* phasic muscle and even lower numbers in the tonic adductor. These results suggest that during long-term emersion, energy is supplied from glycolysis, but also that complete breakdown of glycogen, via the more efficient aerobic

pathways, is limited. The low aerobic capacity of scallop muscle also requires that energy-demanding escape swimming is fuelled by alternative means, mainly from the high energy phosphagen, phospho-l-arginine (de Zwann *et al.*, 1980). The supply of phosphagen is limited, however, which in turn limits the number of consecutive adductions to about 35 (Thompson *et al.*, 1980). Depletion of the phospho-l-arginine pool may therefore explain the rapid decrease in adduction frequency after the first hour of emersion, and suggests that any subsequent adductions are supplied from glycogenolytic energy pathways. Quantitative analysis of muscle-tissue glycogen confirmed the use of this substrate during emersion. The initial muscle-glycogen concentrations recorded in this study agree well with those reported for this species. Comely (1974) determined percentage carbohydrate (which is likely to be almost exclusively glycogen) in Clyde populations of *Pecten maximus* at 10.4 and 4.2% for phasic and tonic adductors respectively. These values were obtained in the same month as the present study's assay, which recorded values of 13 and 8.2% for equivalent muscle types. The slightly higher figures obtained in this study may be related to different assay methods, scallop growth conditions, or perhaps to differences in the timing of the analysis. No actual dates are given in Comely's work, and the glycogen analysis in this study was carried out at the end of June. Comparison with Comely's July value of 12.9% for phasic muscle is much closer and therefore some of the variation may relate to this factor.

Use of glycogen by *Pecten maximus* during emersion is substantial. The concentration in phasic and tonic adductors was reduced by 64 and 29% respectively after 24 hours, by which time valve gaping had occurred. The higher utilization rate by phasic muscle was pronounced during the first 12 hours in air and presumably relates to the energy requirement for adduction, which was most frequent during this period. After 12 hours, the glycogen concentrations in both muscle types were not significantly different from each other (paired t-test, $P > 0.05$), which was also reflected in the PAS-stained tissue (plate 6.5). The breakdown rate of phasic glycogen then decreased up to 24 hours, but, as previously mentioned, by this time gape was maximal and adduction was very infrequent despite the fact that large quantities of glycogen remained. This supports the earlier conclusion that the energy substrate is not fully depleted and thus the first hypothesis appears invalid. This view is, perhaps, also supported by the findings of Hummel *et al.* (1989) who concluded that *Mytilus edulis* and *Cerastoderma edule* did not die during emersion on account of low energy reserves, but rather as a consequence of the metabolic pathways they employed which resulted in the accumulation of high concentrations of acid end products.

Based on previously determined respiratory data and the quantity of glycogen used during emersion, approximate values of energy consumption by *Pecten* muscles may be derived (de Zwann & Wijsman, 1976). These calculations suggest that, depending on the energy-production efficiency of the glycogenolytic pathway used, the scallop is capable of providing between 63

and 134% of the resting aquatic energy requirement during the course of the emersion. This does not, however, take into account the increased energy demand from adduction and the higher value assumes complete anaerobic breakdown of one mole of glycogen to propionate with a maximum possible ATP production of 6 moles. Considering the more probable situation, of an increased energy requirement and a limited anaerobic capacity for energy production, it seems likely that an energy deficient state will develop during emersion. The second hypothesis, that insufficient energy can be produced from available metabolic pathways, was investigated using the Adenylate Energy Charge parameter, i.e. the ratio between the nucleotides, ATP, ADP and AMP, which indicates the amount of metabolic energy available from the adenylate pool. Since its proposal by Atkinson & Walton (1967) many studies have investigated, or used, this ratio as a general indicator of biological stress. The fundamental importance of ATP and related nucleotides in energy metabolism means that changes in relative nucleotide concentrations promote or inhibit many enzymic reactions involved in the regulation of metabolism, including ATP (energy) producing or utilizing reactions (Atkinson, 1968). The optimum condition for a cell or tissue is a high proportion of ATP, which is required for all energetic processes, and therefore the feedback mechanism acts to maintain the ratio of nucleotides (AEC) between 0.9 and 0.6, thus ensuring a high ATP concentration in optimum conditions and ATP conservation or generation in sub-optimal conditions (Wijsman, 1976). Although some inter- and intra-specific variation in AEC may occur, the calculated values for animals under normal conditions are always within this range (Chapman *et al.*, 1971; Beis & Newsholme, 1975). In addition, several other characteristics identify it as a useful condition indicator under certain circumstances (Ivanovici, 1980a).

1. A reduction in AEC is consistent with environmental conditions that may jeopardise survival.
2. AEC has never been shown to increase in response to stress.
3. The response to stress is rapidly reflected in the energy charge.

The chief disadvantages of AEC, particularly as a tool for population or chronic stress monitoring, relate firstly to the fact that only resistant or healthy animals will occur in field populations, since low AEC animals will be dead (Verschagen *et al.*, 1985), and secondly to the relative difficulty of sample preparation and analysis (Ivanovici, 1980a). ATP is particularly labile and the tissues require rapid fixing and careful processing if accurate AEC values are to be obtained. The method employed in this study used HPLC rather than an enzymic assay (Ivanovici, 1980b) which, although highly sensitive to ATP, has the disadvantage of secondary calculation of ADP and AMP from the ATP values. Chromatographic analysis, however, identifies each nucleotide separately from samples prepared by acid extraction, although the actual method adopted may vary (Wadley *et al.*, 1980; Moal *et al.*, 1989).

The present study found mean ATP concentrations of 48.6 and 13.7 $\mu\text{mol. g}^{-1}$ (dry weight) in phasic and tonic adductors respectively. These values compare quite well with equivalent results obtained by Beis & Newsholme (1975) of 6.63 $\mu\text{mol. g}^{-1}$ (wet weight) for phasic adductor (this study; 9.72 $\mu\text{mol. g}^{-1}$, assuming dry weight is 20%) and Ansell (1977) who reported 1.75 and 0.65% ATP of dry tissue weight in phasic and tonic muscles (this study; 2.46 and 0.69%). Values for ADP and AMP were also comparable. The results obtained in the cited studies used enzymic determination and therefore the slight differences may be attributable to the different assay methods.

Statistical analysis (ANOVA) of the data indicated that there was no overall change in the adenylate pool, i.e. the total of all the related nucleotides. Hence, changes in one nucleotide resulted in a concomitant change in another. This is clearly demonstrated in the chromatograms (Fig. 6.4 a,b), which show the formation of ADP and AMP as ATP is broken down. Not surprisingly, AEC decreased over the 72 hour emersion period, since it is a direct reflection of available metabolic energy, i.e. ATP, and indicates that the scallops are exposed to conditions which prevent maintenance of energy balance in the long term. It is interesting to note, however, that during the first 12 hours of exposure energy charge did not change in either muscle type, which may relate to the high glycogen utilization rates, in conjunction with maintenance of some aerobic pathways which produce more ATP per glycogen molecule. Evidence for this hypothesis comes from the respiratory data (chapter 4), which indicate that some oxygen is still being consumed during this time. In addition, calculation of potential ATP production from the glycogen used suggests that between 127-270% of the resting aquatic requirement could be produced, depending on pathway efficiency. Even accounting for the additional adduction requirement it seems likely, therefore, that over the initial 12 hours the muscles will remain in energy balance. Hence, AEC is maintained. The AEC value of 0.88 for phasic adductor is close to the published values for this species and is broadly comparable with other bivalve adductor muscles (Table 6.3).

Table 6.3 Published values of AEC from bivalves. Values marked * indicate HPLC analysis, other data determined via enzymic methods.

SPECIES	TISSUE	AEC	REFERENCE
<i>Pecten maximus</i>	Phasic adductor	0.94	Beis & Newsholme, 1975
<i>Pecten jacobus</i>	Adductor muscle	0.93	Grieshaber & Gäde, 1977
<i>Aequipecten opercularis</i>	Adductor muscle	0.93	Grieshaber, 1978
<i>Placopecten magellanicus</i>	Phasic adductor	0.94	Livingstone <i>et al.</i> , 1981
	Tonic adductor	0.85	Livingstone <i>et al.</i> , 1981
<i>Mytilus edulis</i>	Posterior adductor	0.91	Wijsman, 1976
	ABRM	0.87*	Gies, 1988
	Posterior adductor	0.76	Beis & Newsholme, 1975
<i>Crassostrea gigas</i>	Whole animal	0.73*	Moal <i>et al.</i> , 1991

There is clearly some inter- and intra-specific variation which may be due to either natural differences or the analytical method employed, although most fall within the range 0.8-0.9 which is indicative of a stable and viable condition (Chapman *et al.*, 1971). Ivanovici (1980a) went further in associating energy charge with environmental and physiological condition. Values between 0.75 and 0.9 suggested optimal survival conditions with high growth and reproductive rates. An energy charge between 0.5 and 0.75 indicates partial stress conditions, with growth and reproductive rates reduced or completely inhibited. Below a value of 0.5, the environmental conditions are severely limiting, resulting in no growth or reproduction and an overall loss of viability, eventually leading to death. This situation is irreversible, even after return to optimal conditions. Although this is a general guide, with variation occurring depending on species, type of environmental stress and animal acclimation, research work covering a wide range of organisms and conditions have supported such a scheme and found it to be useful in specific applications.

Following the initial maintenance of energy charge by the scallop, AEC decreased significantly between 12 and 48 hours and reached values of 0.4 and 0.36 in phasic and tonic muscle respectively. This rapid decrease in muscle energy charge is similar to the response shown by *Mytilus edulis* during experimental emersion (Wijsman, 1976), which was thought to relate to the change over from aerobic to anaerobic metabolism. This interpretation could also apply to *Pecten maximus*, since both oxygen consumption and glycogen utilization declined over this period and, as indicated previously, calculated potential ATP production does not appear sufficient to maintain long term energy balance via predominantly anaerobic metabolism. In contrast to this study, however, the AEC of *M. edulis* did not decline below 0.62, which is

consistent with the theory of Atkinson (1968) which predicted that within this range ATP regeneration would still be enhanced, whereas processes resulting in ATP utilization (or storage) would be inhibited. Such a strategy would be a useful adaptation to sustain long periods of anaerobiosis during emersion. In some bivalves the anaerobic generation of ATP from glycogen, while resulting in a higher net yield than the equivalent vertebrate pathway, is still considerably less than that derived from complete carbohydrate oxidation (Wijsman, 1976). Consequently, during anaerobiosis in air, *Mytilus edulis* must reduce its overall metabolism if it is to survive long emersion periods and remain in energy balance (de Zwann & Wijsman, 1976). Based on the evidence of AEC and the results of aerial respiration and PO_2 measurements (chapter 4), it appears that *Pecten maximus* maintains ATP levels during the first 12 hours of emersion with an increasing reliance on anaerobic metabolism as oxygen uptake declines. The combined metabolic capacity for energy production appears to be sufficient to keep the AEC constant over this period, but diminishes with increasing use of anaerobic pathways, resulting in a decrease in the AEC of the scallop to a level considered to be irreversible and fatal. The ability to generate sufficient energy from anaerobic metabolism may simply be inadequate in the scallop, or it may perhaps decrease over time as accumulation of end products reach levels which damage cell function and prevent biochemical processes. Further evidence for this terminal state comes from the apparent increase in AEC at 72 hours, a feature also recorded for *M. edulis* after 7 days in air (Wijsman, 1976). The energy charge value recorded after that time was identical to that measured in dead animals, an increase of 0.1 over the previously recorded minimum value, and it was suggested that the increase indicated that the mussels had started to die.

It seems likely, therefore, that *Pecten maximus* does use anaerobic metabolism during emersion as a result of a declining oxygen influx caused by gill dysfunction. However, the energy demand, or metabolic rate, is not reduced and may actually be increased by adduction activity, eventually resulting in an energy deficit. Adaptation for energy production and conservation, which is a survival feature of littoral species such as *Mytilus edulis*, appears to be lacking in the sub-littoral scallop.

The final factor which may accelerate deterioration of the adductor muscle and other physiological systems in an emersed scallop is that of intra-muscular metabolite accumulation. The investigation carried out during this study supports the hypothesis that anaerobic metabolism is used to some extent during emersion since acid end products were detected in the muscle tissue. The principal products of bivalve anaerobiosis depend on the species, substrate type(s) and environmental conditions involved, but typically consist of acids, such as lactate and succinate (de Zwann & Wijsman, 1976), amino acids (de Zwann, 1977) and, of particular interest in scallops, the imino acid octopine (Grieshaber & Gäde, 1977; Gäde *et al.*, 1978; Grieshaber, 1978; Fields, 1983). There are a number of potential anaerobic pathways available to emersed marine bivalves and even within a single species, different pathways are used at

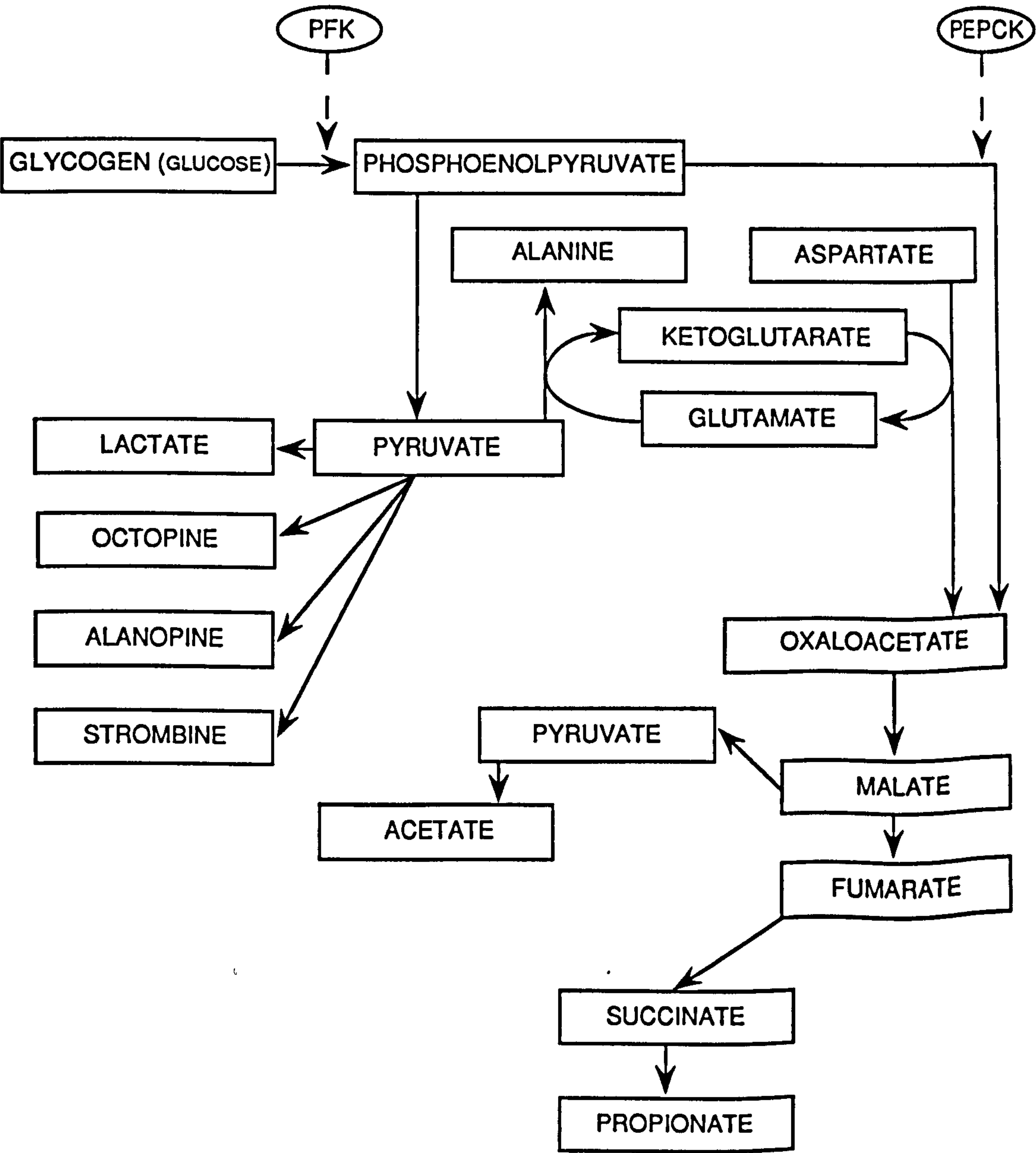
different times and under different conditions, for example during functional or environmental anaerobiosis (Gäde, 1983). Different pathways utilize alternative substrates or produce alternative intermediate and terminal end products (Fig. 6.6). However, the fundamental function of them all is the production of ATP from substrate level phosphorylations, i.e. the transfer of phosphate to ADP from initial or intermediate compounds, and the maintenance of redox balance.

If glycogen is the primary substrate then glycolysis results in the formation of phosphoenolpyruvate (PEP). At this point, depending on specific enzyme activity, PEP may be converted to pyruvate or oxaloacetate. From pyruvate, the classical anaerobic pathway results in production of lactate which is a terminal end product. Alternatives to lactate, utilized by some bivalve species, are the opine compounds alanopine, strombine and octopine. There has been a considerable amount of work done on octopine production in pectinids and its role in anaerobic metabolism. The current view is that it does not play an important part in long term anaerobiosis, but is involved in the maintenance of energy production following burst activity, such as escape swimming (Grieshaber & Gäde, 1977; Gäde *et al.*, 1978). Escape activity, which occurs in response to predators, shows an adduction pattern which differs from the typical emersion response in both intensity (2-3/s) and duration (1-2 minutes) (Gäde *et al.*, 1978). This activity is fuelled initially by phospho-l-arginine which breaks down to form arginine and ATP, and is used until the phosphagen pool is depleted, coinciding with exhaustion. At this point the regeneration of the phosphagen pool requires a period of aerobic recovery in order to return to pre-activity levels. Muscle oxygen concentrations, however, remain low for a considerable time following exertion, and therefore energy supply during this period must come from anaerobic metabolism, principally by the formation of octopine from the accumulated arginine and pyruvate, derived from the stimulation of glycolysis. As arginine phosphate and ATP levels are restored, glycolysis is gradually inhibited and octopine is remetabolised.

Alternatively, if PEP is converted to oxaloacetate, via PEP carboxykinase (PEPCK), then two potential terminal end products, propionate and acetate, may result. The acetate pathway is reached by way of the organic acids, malate and pyruvate, whereas propionate is formed via malate, fumarate and succinate. Succinate itself is frequently accumulated as an end product in many bivalves, particularly during temporary hypoxia, with final conversion to propionate occurring if hypoxia is extreme or extended (Hochachka, 1980).

The final common anaerobic pathway utilises the amino acid, aspartate, as a primary substrate rather than glycogen. Under these circumstances fermentation of aspartate results in the formation of oxaloacetate which in turn enters the routes to acetate or succinate and propionate.

Fig. 6.6 Principal pathways of anaerobic metabolism in bivalve molluscs. Primary substrates are glycogen and aspartate. The enzymes phosphofructokinase (PFK) and phosphoenolpyruvate (PEPCK) are involved in glycogenolysis. See text for discussion.



The described pathways are not mutually exclusive and under changing conditions a number may be used simultaneously or in sequence, resulting in a rather complicated picture. The acid products detected in this study indicate the accumulation of succinate and propionate, although the actual values obtained are higher than might be expected. For example, typical basal concentrations of muscle succinate in other marine bivalves ($50.3 \mu\text{mol. g}^{-1}$ in this study) range from $0.25 \mu\text{mol. g}^{-1}$ (dry weight, converted at 20% of wet weight) in *Placopecten magellanicus* (de Zwann *et al.*, 1980), $1.25 \mu\text{mol. g}^{-1}$ in *Argopecten irradians concentricus* (Chih & Ellington, 1983), 2.1 & $1.3 \mu\text{mol. g}^{-1}$ in *Mytilus edulis* at 10 and 15°C (Widdows *et al.*, 1979; Shick *et al.*, 1983), $3.6 \mu\text{mol. g}^{-1}$ in *Cardium edule* (Widdows *et al.*, 1979), $3.75 \mu\text{mol. g}^{-1}$ in *Geukensia demissa granosissima* and $9 \mu\text{mol. g}^{-1}$ in *Modiolus squamosus* (Nicchitta & Ellington, 1983). Clearly, the values obtained in this study are high, which may perhaps be explained by factors related to the analysis method. The nature of this investigation, i.e. a general analysis to determine which, if any, acid end products were present following experimental emersion, required the use of a relatively non-specific detection wavelength of 210nm. Different acids have optimum wavelengths for detection which must be determined individually (D.A. Scott, pers. comm.), and, therefore, the adopted method must be a compromise to allow a more rapid analysis of samples containing a mixture of acids. An example of the lack of acid specificity was the fact that a number of amino acids could also be detected using this column, including aspartate and glutamate. In a tissue as biochemically complex as adductor muscle it seems possible that many chemically-related compounds could be detected in the samples, perhaps accounting for peaks which did not correspond to any of the standard acids. Peak separation was also poorer on this column than for the nucleotides and integration of peak area by the software program is likely to overestimate concentrations due to these other compounds eluting close to the standard acids. Unfortunately, constraints of time and instrument availability have made it impossible to rectify this situation, so the data should be treated as indicating relative, rather than absolute values.

In those species which show accumulation of acid end products during hypoxia and emersion, the actual concentrations reached depend upon the species and the length and severity of the period of hypoxia. For example, the tendency of littoral bivalves to seal their shell valves during emersion and to resort to complete anaerobiosis, results in the concentration of succinate in the tissues of *Mytilus edulis* increasing by a factor of 9.7 during 48 hours of emersion (Widdows *et al.*, 1979). Similarly, the sub-littoral species *Modiolus squamosus*, which also seals its shell valves during exposure, showed a four fold increase in succinate concentration during a 7 hour emersion (Nicchitta & Ellington, 1983). In contrast, the same study investigated the air breathing species, *Geukensia demissa granosissima*, which also showed an increase of similar proportions (x3.7) during the first 4 hours in air, but thereafter succinate concentrations decreased rapidly to pre-emersion levels, indicating only temporary anaerobiosis. Another valve-gaping, air-breathing, intertidal bivalve, *Cardium edule*, also showed a modest change in

succinate concentration from 3.6 to 7.85 $\mu\text{mol. g}^{-1}$ (x2.2), which again peaked within the first 6 hours and declined below control values after 48 hours in air (Widdows *et al.*, 1979). These examples serve to illustrate the differences between species capable of oxygen uptake during emersion, and which rely on largely aerobic metabolism, and those which tend to decrease metabolism and become fully anaerobic. In *Pecten maximus* the absence of significant changes in intermediate anaerobic end products in the adductor muscles during the first 12 hours of emersion indicates that metabolism is probably largely aerobic during this time, a view supported perhaps by the maintenance of energy charge over the same period. The tonic muscle does, however, show an increased malate concentration, which may indicate a greater reliance on anaerobic pathways early on. The relatively small increases in muscle succinate after 24 hours, by a factor of 1.37 and 1.79 in phasic and tonic respectively, indicate that, in comparison with the previously mentioned species, the scallop does not, or cannot, make substantial use of anaerobic pathways despite a significant reduction in oxygen consumption and internal PO_2 (chapter 4). The decrease in energy charge over this time is also indicative of a poorly adapted anaerobic capacity to generate the required amounts of ATP. After the 24 hour peak of anaerobic activity, succinate and malate concentrations tended to decrease, indicating that this form of metabolism was not maintained, since it is unlikely that they would be excreted. High concentrations of the terminal end product, propionate, were found to occur 12 hours after high concentrations of the other main products, a feature noted in earlier anaerobic studies (Widdows *et al.*, 1979; Shick *et al.*, 1983). This results from the final conversion of the accumulated intermediates and indicates the severity of the anoxia which the scallop is suffering after 72 hours in air (Hochachka, 1980).

The higher concentrations of end products in the tonic muscle indicate that this tissue relies on anaerobic metabolism much earlier and to a greater extent than the phasic adductor. This may be explained by two factors. Earlier work showed that mitochondria were less abundant in the tonic muscle and thus limited the aerobic capacity, since the main energy generating cycles of this pathway occur exclusively in these organelles. Additionally, in the scallop *Placopecten magellanicus*, the tonic muscle has less than 25% of the arginine phosphate levels found in the phasic muscle (de Zwaan *et al.*, 1980), therefore the energy contribution from this source is limited, and consequently energy generation in tonic muscle relies on glycolysis. Assuming a similar situation exists in *Pecten maximus*, then with few mitochondria and limited oxygen uptake during emersion, only anaerobic pathways are available and must be utilized from the onset of exposure.

The acid compounds detected indicate use of glycogen or aspartate fermentation to some degree, although without further analysis it is not possible to determine the contribution that the octopine pathway might make. Gäde (1983) considered that use of glycogen and aspartate substrates, with a succinate or propionate terminal end product, was used during environmental

anaerobiosis, but functional anaerobiosis, i.e. a result of muscle activity, utilised arginine phosphate and glycogen and resulted in the production of opines. Since the present situation appears to involve both a functional and environmental component it is possible that both are used simultaneously or sequentially.

Although previous studies have shown that *Pecten maximus* appears to conform to the hypothesis that octopine is primarily produced and utilized during recovery after escape swimming (Gäde *et al.*, 1978), its role during emersion remains unclear. Interestingly, in the closely related species, *Pecten jacobaeus*, octopine was not detected following the typical adduction behaviour in air, but was found to accumulate after adductions induced by sea-star extracts (Grieshaber & Gäde, 1977). This example illustrates that the latter, escape-type adductions, are supported by anaerobic pathways and the less frequent emersion adductions have a sufficient recovery interval to allow maintenance by mainly aerobic means, at least in the short term. This scenario would also fit the observations made during the present study. In a wider context, however, octopine accumulation would tend to be less significant in physiological disruption, compared with other acid end products, due to its reduced effect on pH and osmotic balance (Fields, 1983).

The actual pathways involved in the production of succinate and propionate are also rather difficult to determine without analysis of aspartate concentration changes and the relative activities of the required enzymes, e.g. PEPCCK. Studies of anaerobiosis in the scallop *Placopecten magellanicus* have produced conflicting results with some workers favouring a glycogen fermentation route (O' Doherty & Feltham, 1971) while others support an aspartate pathway (de Zwann, 1977). This study did record aspartate concentrations in phasic adductor of $61.5 \mu\text{mol. g}^{-1}$ dry weight (compared with $40\text{--}60 \mu\text{mol. g}^{-1}$ in oyster heart) (Hochachka, 1980) and therefore this pathway is potentially viable during emersion. However, in the context of the present study these arguments are secondary since the important points are that potentially damaging acid end products do accumulate and, from the energy charge analysis, it is clear that the pathways involved are not sufficient to prevent a significant and ultimately fatal deterioration in energy balance.

In addition to deficient energy production, a further consequence of sustained anaerobic metabolism is the effect of accumulated end products on the pH balance of the muscle tissue. A detailed study of acid-base balance in emersed *Pecten maximus* carried out in conjunction with the present work, found that the main cause of the substantial haemolymph acidosis was respiratory in origin, although between 24 and 72 hours there was an indication of a slight metabolic component (Duncan *et al.*, in prep.). Metabolic acidosis results from accumulation of the acid products of anaerobic metabolism and their small contribution supports the conclusion that the use of anaerobic pathways is limited in comparison with other bivalves. The systemic

acidosis was therefore caused by inability to remove CO₂, although absence of significant concentrations of metabolic acids in the haemolymph does not preclude a localised effect in the tissues in which they form. Work by Ellington (1983b) on the mussel species, *Mytilus edulis* and *Geukensia demissa* recorded pH decreases in posterior adductor preparations of 0.26 and 0.39 respectively and, although these changes reflected relatively low rates of anaerobic metabolism, they were considered to be sufficient to affect muscle metabolism. This factor would be particularly relevant if the organic acids behave like octopine which is not translocated between muscle types or from the muscle to other tissues (de Zwann *et al.*, 1980). Histochemical staining indicated that both the glycogen substrate and mitochondria were concentrated around the margins of muscle fibres, a pattern shown in other muscle tissues (Vander *et al.*, 1985; Fowler & Neil, 1992). Accumulation of the acids will therefore occur in a relatively confined area adjacent to the individual muscle fibres, tending to concentrate cellular imbalances around the contractile mechanism itself. The implications of pH imbalance have been discussed in chapter 4, although a feature of potential importance to maintenance of muscle tension may be the relationship between pH and AEC. MacFarlane (1981) reported that low pH resulted in a reduction of energy charge in the tissues of the teleost *Fundulus grandis*. In common with this study, there was no significant change in the total adenylate pool although energy charge decreased from 0.83 to 0.55 after exposure to pH 4 for 96 hours. Packer & Dunson (1970; 1972) reported an additional effect of low pH on physiological equilibrium, that of a reduction in the sodium ion concentration of body tissues. MacFarlane (1981) proposed that this observation may be explained by Na⁺ ion pump failure as a result of insufficient ATP availability.

If a similar effect occurs in the adductor muscles of *Pecten maximus*, i.e. pH changes affecting sodium channels, then the concentration of acid-end products around each muscle fibre could have a profound effect on action potential propagation. Ultimately, this would lead to failure of the contractile mechanism and loss of muscle tension. Permanent gape would result if muscle cells and the contractile mechanism became irreversibly damaged and observations suggest that this occurs between 12 and 24 hours after initial emersion. This situation is not rectified following return to aerated sea water. The slow increase in valve gape throughout this period suggests a gradual accumulation of acid products, resulting in a gradual decline in muscle tension. This also supports the earlier hypothesis that anaerobic pathways predominate earlier in the tonic adductor, since it is this muscle which maintains valve closure.

In order to answer the original questions of whether gape causes death, or is gape the result of the factors which cause death, it is also necessary to consider the earlier work which investigated the effect of enforced shell closure on emersion survival (chapter 3). During this experiment elastic bands prevented active adduction and shell gape in one group, although gape did occur if the bands were removed during the emersion period. No differences in survival were

found between closed and freely adducting scallops, and therefore these results indicate that if the processes causing gape were still occurring but, that no survival differences occurred when gape was physically prevented, then it was not the actual gaping which caused death. The fact that gaping occurs, even in the absence of adduction, perhaps indicates that continuous muscle contractions still occur despite the absence of valve movement. This would seem reasonable since the stimulus for contraction, presumed to be low PO_2 , would still prevail. Observations made during this experiment also indicated that the time taken for a maximum gape to develop in the restrained scallops was slightly longer, although actual measurement was not possible due to the experimental conditions. This feature might also be predicted since the low work rate of the 'isometric' type contractions in a closed scallop would require less energy resulting in lower rates of acid accumulation.

The available information indicates that gaping occurs due to deficient energy production and acid accumulation from the metabolic pathways which are used. Aerobic metabolism is limited by oxygen uptake and therefore anaerobiosis occurs. Active adduction results in these conditions developing more quickly, due to higher metabolic demands. Shell gaping also occurs in scallops prevented from valve adduction, since the metabolic processes still continue, although probably at a lower rate. Valve gape is therefore indicative of declining condition and cannot be prevented during emersion, only its development can be retarded. Delaying the onset or extent of gape requires a reduction in overall metabolism or the provision of conditions which enable aerobic metabolism to occur.

In a wider context it is interesting to compare the results of this study with those obtained for *Mytilus edulis* and *Cardium edule*. Hummel *et al.* (1989) found that both species were capable of survival in air for periods of up to 14 days, but that their energy metabolism differed depending on somatic and emersion conditions. In common with this study, however, it was concluded that the bivalves did not die of substrate depletion, but because of a high metabolic acid accumulation. Acid concentrations were high after 4 days at 20°C, up to 275 and 150 $\mu\text{mol. g}^{-1}$ dry weight respectively (converted at 20% of wet weight), although a considerable difference in acid concentration and survival was recorded at 5°C. These results support the conclusions of this present study, that anaerobic metabolites and overall metabolic rate are important for survival during emersion. The work did not, however, determine the significance of energy production on survival ability, which this present study also considers to be important.

Chapter 7 Conclusions and recommendations

7.1. The principal causes of scallop death during emersion

During emersion *Pecten maximus* dies due to progressive respiratory failure, compounded by additional factors which affect cellular function in essential physiological systems, such as circulation, neuro-muscular integration and pathogen resistance.

Initial observations suggested that loss of water from the mantle cavity, due to adduction and drainage at the shell margins, resulted in clumping of the gill filaments. Experimental measurement showed that during the first 8 hours of emersion this caused a rapid decrease in oxygen uptake (by about 60%), which continued to decline over the following days. This effect was also reflected in the oxygen partial pressure of the haemolymph (blood) which decreased by 57% within the first 12 hours of emersion. Additionally, reduced gas exchange efficiency resulted in CO₂ accumulating in the haemolymph rather than being excreted (Duncan *et al.*, in prep.). A low internal partial pressure of oxygen (PO₂) has several important consequences for the scallop. Firstly, internal PO₂ affects heart activity, possibly by limiting available oxygen to the heart muscle itself, and during emersion heart rate decreased substantially. Although this response was only measured over the first few hours in air it would seem likely that any further decrease in PO₂ would cause a further reduction in heart rate (Bayne *et al.*, 1976). Reduction of hydrostatic support for the body tissues, as a result of water expulsion during adduction, may also interfere with heart action and haemolymph circulation if the visceral mass compresses the heart and vessels. This may result in a higher work rate, and hence oxygen demand, by the heart in order to overcome the increased resistance to haemolymph flow. Alternatively, if oxygenated haemolymph is not transported efficiently from the gills then this additional decrease in internal PO₂ will contribute to the feedback mechanism controlling heart rate. Experimental results and observations suggested that complete cardiac failure occurred after about 72 hours in air.

Hypoxia has also been shown to cause increased valve movement or adduction in some bivalves (Miyauti, 1968) and it would seem to be a probable cause of the behaviour shown by *Pecten maximus* during emersion. The principal result of adduction is likely to be an increased energy demand and, hence, an increase in the rate of the metabolic processes involved in energy production. This would result in higher rates of substrate (stored energy reserve) utilization and waste production. The scallop is therefore faced with the resultant problems of supplying the extra energy demand, and with the toxicity of waste accumulation. The second main implication is related to the generation of energy when the most efficient metabolic processes, which require oxygen, are largely unavailable if gas exchange is limited. Previous work has shown that, during emersion, anaerobic metabolism is only able to supply sufficient energy to the littoral bivalve *Mytilus edulis* if the metabolic demands of the organism are substantially reduced (de Zwann &

Wijsman, 1976). It is apparent that a reduction in metabolism does not occur in the scallop and experiments have shown that resorting to anaerobic metabolism does not produce sufficient ATP to meet the energetic requirements of this species during emersion. Consequently, an energy deficient state is reached after 48 hours in air. This is indicated by the adenylate energy charge of the muscle tissue which decreased during emersion by up to 41%. At the values reached, this parameter predicts that physiological failure is inevitable as a result of low energy levels causing cell death (Ivanovici, 1980a).

An additional consequence of using anaerobic metabolism is that the waste products are often acidic (de Zwaan & Wijsman, 1976). In this study malic, fumaric, succinic and propionic acids were consistently detected in the muscle tissues. In sufficient quantities these compounds are likely to enhance cellular deterioration as a result of pH reduction, unless they are chemically buffered. Metabolically derived acids do not appear to be transferred outside the muscles where they are produced, since they do not appear in the haemolymph in high concentrations, even after 72 hours of emersion (Duncan *et al.*, in prep.). The effects of these acids are therefore likely to be confined to the muscle tissues themselves resulting in failure of the adductors and the observed reduction in adduction frequency and increase in valve gape.

Acidification of the haemolymph does occur from a different source, as a result of CO₂ accumulation caused by reduced gas exchange at the gills. The reduction in pH, from 7.6 to 6.6, is substantial and progresses throughout the exposure with no significant buffering by the usual bivalve mechanism of mobilizing shell carbonates (Crenshaw & Neff, 1969; Byrne *et al.*, 1991). A systemic and muscular acidosis will result in membrane disruption, osmotic imbalance and eventual death of cells exposed to such conditions. This in turn will contribute to whole organism death.

Ammonia was found to accumulate in the scallop during emersion, both externally and internally, increasing from less than 5 $\mu\text{mol. l}^{-1}$ to 413.6 $\mu\text{mol. l}^{-1}$ in the mantle cavity, and from 17.1 $\mu\text{mol. l}^{-1}$ to 448.6 $\mu\text{mol. l}^{-1}$ in the haemolymph within 48 hours. Ammonia is the main waste product from nitrogen metabolism in aquatic organisms and is formed under both aerobic and anaerobic conditions, its production being related to the overall metabolic rate (Bayne *et al.*, 1976; Thompson *et al.*, 1978, 1980). When immersed in water the toxic effect of ammonia is reduced by rapid diffusion, but during emersion no rapid removal mechanism exists and consequently the harmful effects are significant. Ammonia is known to particularly affect the respiratory organs of aquatic animals (Jeney *et al.*, 1992) and electron microscopy studies illustrated progressive separation of scallop gill filaments and gill epithelial damage during an experimental emersion period. Observations made during the experimental determination of ammonia toxicity indirectly confirmed ammonia as the cause of this gill damage and also showed that a concentration of 1858 $\mu\text{mol. l}^{-1}$ was sufficient to cause the death of half an

experimental group of scallops within 96 hours. The concentrations recorded in emersed animals after 48 hours are within the range estimated to cause sub-lethal effects and significant long-term mortalities (Burrows, 1964; This study). It is likely that epithelial cells and the cells lining the heart and circulatory system will be affected by these concentrations and that this damage will further contribute to the respiratory problems described earlier, in addition to causing permanent damage to the gill structure itself.

These results suggest, however, that ammonia is only a contributing factor to the short-term survival of commercial-size scallops during transit but, because ammonia is considered to have a greater effect on juvenile organisms, its effects may be more important during the transit of spat and juvenile scallops from collection and ongrowing sites. In areas of abundant spat fall, for example the waters around Skye and Lochalsh, a significant proportion of some producers' income is obtained from the sale and shipment of 4-5 month old scallops. *Pecten maximus* spat may be sold for 2 and 5 pence each, for 10 and 25 mm individuals respectively, and quantities of up to 100,000 may be transported for up to 600 miles (15 hours) within Britain, and as far as France and Spain in some cases. These quantities represent a substantial investment and, because the spat are intended to be ongrown at the destination, it is important to minimise stress and avoid sub-lethal damage which may manifest itself as low growth rates or poor survival during culture. Ammonia accumulation appears to be potentially important in this respect, particularly when the large number and vulnerability of the stock is considered. Control of ammonia concentration within a water re-circulating transit system could perhaps be achieved by provision of an in-line, ion-exchange filter or reduction of the more toxic NH_3 fraction by manipulation of pH. This system will be discussed in more detail later in this chapter.

These factors appear to constitute the basic physiological problems encountered by an emersed scallop, but they may be significantly enhanced by additional features of the emersion environment.

In a natural environment, water flow induced by the beating of cilia is a vital component of normal physiological function in the scallop since it is central to both feeding and respiration. During emersion, the absence of water flow not only affects these processes, but also limits the ability of the animal to remove harmful waste products and control the natural microbial population. Rapid proliferation of bacteria during emersion, of up to 10 fold per day in the static mantle fluid, results in production of metabolites, such as ammonia, which add to the effect of those produced by the scallop itself. Additionally, bacteria also release tissue-damaging enzymes as part of their nutritional cycle which results in cessation of cilia movement and the breakdown of epithelial cells and membranes (Nottage *et al.*, 1989). Once tissue erosion begins, further bacterial proliferation and invasion will occur in the presence of large concentrations of dissolved nutrients. The overall effect of bacterial activity on the emersed bivalve was

demonstrated by the application of antibiotics, which resulted in a significant improvement in scallop survival by up to 20 hours.

The effect of environmental conditions during emersion is also an important factor in determining the duration of scallop survival. Temperature influences the metabolism of the scallop directly, with higher temperatures leading to higher energy demands and increased production of toxic metabolites. This was reflected in experimental survival trials since mortality showed a progressive increase as the emersion temperature was raised from 5 to 20°C. Available metabolic pathways result in deficient energy production under a 10°C regime, and at 20°C, when the metabolic energy requirement is greater, an energy deficient state will occur more quickly. Poorer survival at higher temperature is, therefore, a result of more rapid cell death as energy levels decline faster, toxins accumulate quicker and pH changes occur more rapidly. Higher temperatures also accelerate bacterial growth and consequently their contributions to cell deterioration occur more quickly.

Reduced humidity is also a significant factor in survival since a 25% decrease in relative humidity resulted in a 27% decrease in survival duration. The principal causes of death from desiccation again relate to a deterioration in the gas-exchange surfaces, and the resultant problems of pH and energy production, in addition to direct water loss from exposed tissue surfaces which causes cell death through osmotic imbalance.

Under natural emersion conditions, such as tidal exposure, desiccation of bivalves may be enhanced by shell valve gaping (Lent, 1968; Coleman, 1973), although this can be avoided during transit if appropriate packing and humidity conditions are observed. Gaping does, however, have an important implication for marketing since it is commonly used to assess product quality. Experimental investigations indicated that gape is caused by low ATP levels, toxin accumulation and probable pH reduction as a result of adopted metabolic pathways. Adduction during emersion caused permanent gape after approximately 12 hours, although gape also occurred when valve movement was prevented. It therefore appears that these detrimental physiological processes occur inevitably, but additional activity increases metabolism and therefore results in the acceleration of gape.

In summary, scallops die in air due to reduced gas exchange. This results in a systemic oxygen deficiency and accumulation of CO₂ with a consequent resort to anaerobic metabolism and significant decrease of haemolymph pH. The anaerobic pathways employed by this species do not generate sufficient quantities of ATP (energy) to maintain viable function. This may be related to the relatively high energy demand required to sustain adduction in air, or, perhaps more probably, to a gradual inhibition of energy-producing biochemical reactions due to the pH imbalance for example. Additionally, over an extended period, the anaerobic energy generating

capacity of scallops may be limited by low activities of necessary enzymes, such as PEPCK (de Zwann *et al.*, 1980), and the low mitochondrial numbers would limit the rate of the terminal substrate phosphorylations. The energy deficient state, and death, occurs more rapidly if metabolism is increased by activity or exposure to higher temperatures. End products of anaerobic metabolism contribute to acidification mainly where they are generated and this localization, coupled with ATP depletion, is considered to cause muscle failure and subsequent shell gaping. Shell gape occurs more rapidly if these conditions are enhanced by the rapid adductions characteristic of scallop emersion. The onset of gape does not appear to be significantly related to survival duration under high humidity conditions but is an important quality control feature of the live scallop market. Metabolically derived ammonia and uncontrolled bacterial growth cause tissue damage and both effects will be enhanced by higher temperatures. The decrease in haemolymph pH, caused mainly by CO₂ accumulation, will significantly affect cellular function and hence overall physiological integration. Scallops do not mobilise shell carbonates in order to neutralise this acidosis.

The combined effect of these factors are more damaging to sub-littoral scallops, which lack significant adaptation to emersion stresses, than to littoral species which survive well during transit. These emersion-tolerant bivalves, in contrast, may utilize atmospheric oxygen more efficiently, reduce their metabolic demands, have a greater capacity for anaerobic energy production and end-product tolerance and are able to minimise pH changes by means of efficient buffering mechanisms.

7.2. Recommended changes to current post-harvest practice

Methods of reducing physiological disruption, and hence improving survival, are clearly limited to manipulation of transit conditions since the morphology and physiology of the scallop is only adapted to function under fully aquatic conditions. The most favourable transit conditions will therefore be those closest to this condition, combined with reduction of controllable stress factors.

The current practice of chilled transport is clearly beneficial and acts to reduce metabolic demands and limit bacterial growth. The actual temperature used is a compromise between minimum metabolism and low temperature damage and lies between 2 and 4°C. Reduction of temperature stress between harvest and transit must also be considered, particularly since this period may be up to 8 hours. This interval may be even more critical if adduction also occurs since this would again tend to increase metabolism and initiate the energy reduced state and end-product accumulation associated with anaerobiosis. It is therefore proposed that immediately on harvesting, scallops are stored in a large volume tank of fresh, aerated sea water. This practice

would reduce temperature stress, allow maintenance of gas exchange, avoid anaerobiosis and acidosis and delay uncontrolled bacterial growth. Adduction activity would also be minimised, although prevention of activity associated with disturbance could be accomplished by enforced closure with elastic bands. The reported practice of sea-water hosing after harvest may temporarily overcome some problems, but adduction, gill collapse, desiccation and temperature stress would still occur and the stressful, and potentially damaging, consequences of regular, high-pressure hoseings are unknown. Retention of scallops in water while at sea would require only a limited number of moderately sized tanks (for example 100-150 litre volume), and less attention than regular hosing would demand. The true feasibility and benefits of this practice could only be assessed by appropriate field trials.

Unfortunately, land transit of scallops using a similar method has been discounted by industry due to the unacceptable cost of water carriage and therefore alternatives must be sought. Maintenance of maximum humidity and minimum temperature are vital, and constitute the basis of current practice. By themselves, however, they are insufficient to prevent the main problems developing. The obvious compromise is the misting system which, although developed for crustacean transport, has been shown by this study to significantly improve scallop survival. The operating principal is to provide a sufficient 'flow' of water to maintain gill function and prevent faecal and metabolite accumulation. Related problems are consequently avoided. The present study used ambient temperature ($9.5 \pm 1.5^{\circ}\text{C}$) for the survival trial but, in combination with a refrigerated vehicle, the usual practice for crustacean shipment, water temperature could be reduced and survival may be further improved. The potential limitations of the system relate to the volume of water required to maintain a large number of scallops and how to prevent toxification of this reservoir during long transit periods. The two problems relate respectively to the different misting options available. The most frequent practice for crustacean transport is to gradually expel the misting water during transit, which although removing the requirement for water purification, obviously requires a greater reservoir volume. The volume required would increase with transit distance and consequently reduce the financial incentive due to the cost of additional fuel consumption and carriage of perhaps several thousand litres of water. The economics are viable for crustacean transit, on account of their high premium, but separate assessment would be required for intermediate value products such as scallops. Alternatively, a smaller reservoir volume, up to 500 litres, may be recycled but would require water purification during transit. The Seafish Industry Authority unit at Ardtoe, which currently uses a misting system to transport scallop spat, have investigated the use of a biological filter to control ammonia build up. This was, however, found to be impractical and at present the maximum transit time for 25mm spat is approximately 15 hours. Alternatively, use of an ion-exchange filter combined with a UV water steriliser to regulate bacterial growth could realise the full potential of this system.

Techniques employed for transport of other commercially important species may also be used to contribute to survival improvement of scallops. Regular transit of teleost brood stock involves carriage in small volumes of water for periods of several hours. The relatively more complex physiology of vertebrates, and consequently greater susceptibility to stress, requires careful preparation and transit procedures, although the problems of respiration, energy expenditure, acidosis and bacterial contamination and infection are similar to those encountered in scallops. The additional complication of fish transport, as with the transport of scallop spat, is that the animal must continue living after delivery. Prior to transit the fish are exposed to a 5°C increase in water temperature for between 24 and 48 hours. The fish are not fed during this time, so the increase in metabolism utilises any remaining food in the digestive system. This results in an absence of faecal production during the transit period and additionally reduces the level of gut flora if the re-circulating water is sterilised. This procedure has some characteristics of the standard bivalve depuration method. Fish transport occurs at 4°C which acts to reduce metabolism and so limit waste production and reduce the requirement for energy production. The final application is the use of a buffered transit medium which consists of 3.2ml HCl (s.g. 1.16g/l) and 7g Tris hydroxymethyl methylamine (tris buffer) per litre of sea water, previously sterilised via a 0.2µm cartridge filter and UV exposure. This solution acts to offset pH changes, which have also been shown to damage teleost gill and tissue epithelia. Use of this procedure in scallop transit would seem plausible since the physiological basis of the method is equally applicable to invertebrates and the problems to overcome are similar. Substitution or incorporation of the buffer solution into a misting chamber reservoir could potentially offset the absence of any significant buffering mechanism in the scallop. Additionally, although these compounds are unlikely to pose any human health risk at the concentrations used, it would be necessary to determine their persistence in a potential food product. Adoption of some or all aspects of this method, and the practicality and benefit of its application to scallops destined for consumption or ongrowing would again require assessment by industry.

The final recommendation, which would apply only to the transport of scallops for ongrowing, re-seeding or broodstock provision, involves the use of antibiotics to control the effects of bacterial proliferation. Food quality regulations are likely to prohibit the use of such compounds in products destined for human consumption. Bacterial control methods acceptable for a live food product must rely on pre-transit depuration, low-temperature storage, the potential use of CO₂ gas (chapter 5) or conditions, such as misting or emersion, which allow the scallop to maintain its natural control mechanisms. Antibiotics, usually penicillin and/or streptomycin, have been effectively used for prolonging shellfish transit life, particularly for spat and juveniles which tend to be more susceptible to bacterial action.

Avoidance of desiccation is also essential for small scallops, although the most common practice is damp packing rather than full immersion which otherwise may be a more viable

proposition for the small, high value spat. Since juveniles are generally more severely affected by adverse conditions than adults it is perhaps surprising that emersed transport is possible at all. It has been suggested that the ability of juveniles to survive emersion relates to their surface area to volume ratio which allows more efficient gas exchange relative to their metabolic rate, and consequently they avoid the rapid occurrence of the physiological changes. The improved gas exchange efficiency has also been utilised to improve survival by providing an oxygen rich transit atmosphere which the juveniles are better able to exploit. This advantage is lost in larger scallops, as shown in this and other studies, and is therefore not applicable to market sized adults.

7.3. Further work

During the course of this study a number of aspects were not completely resolved and several new areas of investigation were suggested.

Foremost among the matters deserving further work is the complex nature of anaerobic metabolism during emersion. The results from this study clearly indicate that anaerobiosis does occur, although this preliminary work did not investigate all the potential metabolic pathways available and, while this would involve a comprehensive and complex analysis, the results may be of considerable comparative interest. In addition, although it also seems that muscle failure probably results more from energy deficiency rather than the accumulation of anaerobic end products, it would be of interest to obtain values for intra-muscular pH in order to determine how important these acid compounds are. As mentioned in the discussion (chapter 6) metabolic acidosis can result in low pH in more anaerobically dependant species, although over an emersion lasting 48-72 hours this may be equally significant in *Pecten maximus*.

An aspect of scallop muscle physiology which was not fully explained during this study was the apparent absence of a more defined seasonality to changes in the muscle glycogen content. The available literature suggests that the observed seasonal fluctuations in scallops are closely linked to feeding and spawning and, since there is considerable variation in these influencing factors, even within the coastal waters of Britain, it is possible that the trend observed in this study relates to the suspended-culture conditions of the experimental scallops. A more extensive study, including comparisons with 'on-bottom', stock, may therefore clarify this point.

Additional work, which may provide further insight into the physiological changes and subsequent effects, might include measurement and comparison of pre- and post-emersion ammonia production rates. This study provided data on ammonia accumulation rates and, therefore, in addition to providing a further indicator of general metabolism, a more

comprehensive picture of the nitrogen cycle might clarify the conditions which maximize breakdown or minimize the fraction of the more harmful end products. During this study microscopic examination of tissue damage concentrated on the external deterioration of gill tissue. An investigation of other tissues, particularly those on the internal surface of the heart and circulatory system, may suggest a more comprehensive mechanism for the actual processes of cellular destruction and determine the effects of the high haemolymph ammonia concentration. Equally, comparison with the effects on juveniles or spat may prove beneficial if the mass transit of these stages is further developed.

Bacterial control is problematical if the live scallop is destined for human consumption. The potential methods discussed may be most beneficial if they could be specifically directed at the most damaging bacteria. Identification of the most active enzyme and ciliostat producers, other than vibrios and aeromonads, might suggest the most efficient control method since their effect may be to bias the dominant genera in favour of less damaging groups. For example, CO₂ is more effective on gram negative than gram positive bacteria (Clark & Takács, 1980). A further point raised in discussion (chapter 5) was the effect of CO₂ on scallop physiology if the gas was employed for bacterial control. Given that scallop gas exchange is compromised during emersion, the presence of high CO₂ levels may not be as harmful as might be considered. Formation of carbonic acid in mantle water or on moist tissues may, however, offset any benefit derived from its additional properties. Its potential requires further investigation.

The final suggestion for further work relates to the possibility of inducing 'catch' in the tonic adductor muscle during emersion. This state is utilized in the scallop in response to some predators, and experimental observations suggested that it may also occur infrequently during emersion. The most common emersion behaviour involved adduction, which, it was proposed, resulted in an inability to develop catch. Very occasionally an emerged scallop did not adduct but instead remained tightly closed for several hours, a situation superficially similar to 'catch'. The research effort directed towards the catch mechanism has provided a method for inducing this state in isolated molluscan smooth muscle (Zange *et al.*, 1989). Application of 0.5 mM acetylcholine in an aqueous medium, followed by subsequent washing, has been used *in vitro* and may also be applicable to the live animal. If this method is viable *in vivo*, possibly in combination with shell binding, then many of the physiological changes induced by increased metabolism could be avoided. A feasibility study of this method, or investigation of alternatives, could produce significant improvement in survival or delay the onset of gape.

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APPENDIX 1

Detailed survey results used to determine the present market for live *Pecten maximus*.

The following results were obtained from shellfish producers, fish hauliers, fish merchants, fish processors and fishermen based on, or buying from, the west coast of Scotland. The survey was specifically targeted towards the live scallop market, although the results are also a good indicator of the current nature of scallop aquaculture in Scotland.

It has been noted in the text (chapter 1) that some questions were not fully answered by all contributors, thus, not all percentage values are based on the given sample size. In these cases percentages are based on the total number of responses obtained. Combined values may not add to 100% due to the effects of rounding up or down.

Where absolute values were asked for only the maximum figure is given. Where the sample size was two or less, or single examples were obtained, the response may appear in full.

Producers	Option	% of total (n=10)
Species produced	<i>Pecten maximus</i> only	60
	<i>Aequipecten opercularis</i> only	10
	Both	30
	Princess scallops	0
Production method	Aquaculture only	40
	Diving only	20
	Both	40
Emersion time between harvest and landing	1-2 hours	25
	2-4	25
	4-8	25
	>8	25
Hosing during emersion		25
Conditions of transit	Moist packing	20
	Chilling	40
	Ice	20
	None specified	20
Market	Processed only	11
	Live only	44
	Both	44
Product destination	Local only	33
	Within Britain	22
	Within Europe	44
Sales frequency	Weekly	62.5
	Yearly	12.5
	Special order	25
Transit Packing method	Boxed	20
	Sack (right valve down)	30
	Chill/ice	30
	No special conditions	20
Product assessment	Visual	17
	Gape	33
	Response	0
	All three	50
Maximum transit time	<12 h	38
	12-48	38
	48-60	25
Maximum transit distance		1120 km

Hauliers	Option	% of total (n=7)
Species transported	<i>Pecten maximus</i> only	86
	<i>Aequipecten opercularis</i> only	0
	Both	14
	Princess scallops	0
Transit method	Road	72
	Air	28
Transit Conditions	Vivier	0
	Cascade	0
	Ice	12.5
	Damp	12.5
	Chilled	75
Packing method	Boxed	78
	Bagged	11
	Basket	11
Maximum transit distance		1000km
Maximum transit time	12-24 hours	57
	24-48	14
	48-60	29
Live arrival specified		100
Delivery to:	Market	0
	Parent company	40
	Retailer	20
	Distributor	40
Destination	Northern France	43
	Central France	29
	Holland	29
	Belgium	14
	Northern Germany	14
	Spain	43
	Portugal	14
Delivery frequency	Weekly	71
	More than once /week	29
Quantities per week		200-250kg

Fishmerchants	Option	% of total (n=7)
Species dealt with	<i>Pecten maximus</i> only	57
	<i>Aequipecten opercularis</i> only	0
	Both	43
	Princess scallops	0
If not live why not?	Financial risk	33
	Excess transit weight	67
Proportion	Live	43
	Fresh	29
	Both	29
	Frozen	0
Supplier	Grower	0
	Trawler	86
	Diver	14
	-----	-----
	Other	14 (processor)
Time since harvest/landing	12-24hours	43 (west of Scotland)
	24-48	40 (London & Boulogne)
Quality assessment	Gape	33
	Reaction	50
	Smell	17
Live quantity and frequency		12000/week (London)
		5000/week (to France)
		1000/week (Glasgow)
Delivery conditions	Sack	60 (Domestic)
	Boxed	40 (Continent)
	-----	-----
	Chilled	60
	Damp	40
	-----	-----
	Pre-packed (not live)	20
Shelf life		3-4 days
		7-8 & up to 10 (sic)
Size (shell breadth)		10 (minimum)-15cm

Processors	Option	% total (n=4)
Species handled	<i>Pecten maximus</i> only	25
	<i>Aequipecten opercularis</i> only	0
	Both	75
Obtained from:	Trawlers	50
	Divers	25
	Both	25
Proportion dealt with live	0	33
	<10	33
	90	33
Quantities/week		1250 (live)
		3000 (live)
		72000 (for processing)
LIVE ONLY		
How transported		Boxed & chilled (Also one example frozen in shell & sold as <u>fresh</u> in Spain)
Customer requirements		Absence of gape
Maximum transit time/destination		60 hours/ N.Spain

Fishermen	Option	% total (n=2)
Species caught	<i>Pecten maximus</i> only	50
	<i>Aequipecten opercularis</i> only	0
	Both	50
Sold to:	Processor	100
	Live	0
	Local trade	occasional
Dredge number		6-8
Average catch		300-360/hour
		150-250 by catch /day
Packing at sea		Sack & ice, with no specific orientation
Landing frequency		Every 2 days
Shore transit		Chilled

Appendix 2

Draft copy of the paper;

'Acid-base disturbances accompanying emersion in the scallop *Pecten maximus* (L.)'

In the following paper results of work carried out during this study are presented. Due to its relevance to aspects of the previous work it has been included in draft form. Following final editing and revision by additional authors this paper will be submitted for publication.

Acid-base disturbances accompanying emersion in the scallop *Pecten maximus* (L.).

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Running head: Scallop acid-base

Key words: scallop, emersion, acid-base balance, magnesium

Abstract

The acid-base disturbances accompanying emersion were studied in the scallop *Pecten maximus* (L.). Most of the recorded changes in acid-base and ionic parameters were evident within 2 h of exposure. There was a decrease in haemolymph PO_2 , pH and Mg^{2+} ion concentration and an increase in the concentration of both bicarbonate and Ca^{2+} ions in the haemolymph. All of the changes, with the exception of Mg^{2+} , were comparatively small. The osmotic pressure of the haemolymph did not alter significantly throughout the emersion period. After 72 h emersion, there was significant mortality among scallops. By this time haemolymph PO_2 and pH were extremely low although the concentrations of divalent ions had returned to pre-exposure levels. Haemolymph bicarbonate remained elevated, although only at a concentration twice that of the controls.

Scallop haemolymph displayed a classic respiratory acidosis upon emersion. This was further compounded by a small metabolic component by the time that significant mortalities began to occur. This was evident by the increase in organic acid concentrations between 24 and 72 hours. Although there was a small increase in the concentration of Ca^{2+} ions in the haemolymph this contributed little to ameliorating the acidosis. Some suggestions are given as to the significance of the marked decrease in the concentration of Mg^{2+} ions in the haemolymph. The importance of acid-base disturbances to bivalves during emersion, and its commercial significance, are discussed briefly.

Introduction

An ability to maintain respiratory gas exchange during the period of low tide is a common feature of many intertidal molluscs (Newell, 1973; 1979). For example, trochid species which occur high on the shore exhibit an increase in mantle vascularization compared with those that occur lower down; this feature is associated with an increased respiratory and activity level in the high shore species (Micallef, 1967; Micallef & Bannister, 1967). Similarly the bivalve *Cerastoderma edule* reduces metabolism and air 'breathes' by gaping its shell valves during emersion. By contrast, the closely related, although exclusively sub-littoral species, *C. glaucum* closes its shell valves and relies solely on anaerobic pathways to meet its metabolic demands (Boyden, 1972).

Although lack of oxygen is rarely a problem in air, emersed animals may be faced with greater difficulties in the removal of CO_2 which has potentially serious consequences for the acid-base balance of an animal. Studies of aerial gas exchange by aquatic animals (acute exposure) indicate that, while in all cases, there was an increase in total haemolymph/coelomic fluid PCO_2 , the respiratory acidosis that resulted was either temporary, in groups such as the crustaceans (Truchot, 1975) and echinoderms (Spicer *et al.*, 1988) or sustained in groups such as the annelids (Toulmond, 1973) and bivalve molluscs (Collip, 1920; 1921; Jokumsen & Fyhn, 1982; Booth *et al.*, 1984; Byrne & McMahon, 1991; Byrne *et al.*, 1991).

In this present study we have examined the acid base balance of haemolymph in the scallop *Pecten maximus* (L.) during emersion. *Pecten maximus* is a sub-littoral bivalve which, under normal conditions never encounters aerial exposure. However, knowledge of the survival and physiological condition of this species during emersion is of commercial interest and this work formed part of a wider study of the post-harvest physiology of *Pecten maximus*. This scallop supports a large Scottish fishery and, although most of the landings are processed, a better price is obtained for the live in-shell scallop. This necessitates prolonged periods of post-harvest emersion and, since the visual appearance and responsiveness of the animal are deemed important quality features, data on the physiological disruption caused by emersion may be useful in improving the quality of the product. Such information is also of interest, however, from a comparative point of view as it should allow us to pin point the physiological responses to, and consequences of, emersion which are shared by littoral and sublittoral species and those which are peculiar to littoral species.

Materials and Methods

Maintenance of scallops

Pecten maximus (L.), of shell height 10 ± 1 cm, were obtained from the Seafish Industry Authority Marine Farming Unit, Ardtoe, Argyll, Scotland. Scallops were transported to the Department of Zoology, Glasgow University in double lined, plastic bags, half-filled with sea water, which had been sealed and inflated with oxygen. Upon arrival the scallops were maintained in a number of large tanks in a recirculating seawater aquarium (salinity=32‰).

Pre-treatment of scallops

To ensure access to the haemolymph, a small area of shell (10 x 5 mm), immediately above the pericardium, was removed, using a dental drill. Great care was exercised to ensure that the mantle and pericardium remained intact. The remaining shell section could be quickly removed as and when required, allowing anaerobic samples of haemolymph to be collected directly from the heart. During surgery scallops were wrapped in damp tissue in an effort to reduce water loss and prevent adduction. Scallops were left to recover from post-surgical trauma for at least 24 h before use in any experiment.

Emersion experiment

The effect of emersion on the acid-base disturbances in the haemolymph of individual scallops was examined as follows. Forty individuals were removed from their holding tanks (time 0) and distributed equally (5 per tank) between a number of empty plastic aquaria (10 l capacity). Each tank was then covered with tissue paper which had been soaked in sea water. This ensured the maintenance of a high relative humidity in the tank. Five individuals were removed at each time interval viz. 2, 4, 8, 12, 24 and 72 h after emersion. Haemolymph was extracted using a 1 ml syringe, the needle of which was inserted through the pericardium, directly into the ventricle. Approximately 300-600 μ l of haemolymph was obtained from each animal. Due to a restriction on animal numbers, control individuals, removed from the holding tanks, were sampled only at 0 and 72 h.

Measurement of haemolymph gases and pH *in vivo*

Measurements of haemolymph gases (PO_2 and PCO_2) and pH were made within 30 s of the sample being extracted from the animal. The *in vivo* pH was obtained by drawing samples of haemolymph (45 μ l) into the microcapillary pH electrode of a BMS II (Radiometer, Copenhagen) which was connected to a Corning 255 ion analyser pH meter. The total CO_2

content of the haemolymph (CCO_2) was determined in duplicate 45 μl samples using the method of Cameron (1971). The total O_2 content of the haemolymph was determined by injecting a 150 μl sample into a thermostatted water jacket (10°C), containing an oxygen electrode (Radiometer 1302, Copenhagen), coupled to an O_2 meter (781 Strathkelvin Instruments, Glasgow) and chart recorder (BBC SE120). The remaining individual samples of haemolymph fluid were then frozen at -20°C until further analysis could be carried out.

Chemical analysis of haemolymph

Haemolymph samples were defrosted at room temperature (20°C) before the following analyses were carried out. The concentrations of Mg^{2+} and Ca^{2+} ($[\text{Mg}^{2+}]$ and $[\text{Ca}^{2+}]$ respectively) in appropriately diluted haemolymph were estimated using atomic absorption spectrophotometry (Philips PU9200). Lanthanum chloride was added to each of the dilutions used in the determinations. The total protein content of the haemolymph was quantified by measuring the absorbance of untreated haemolymph (1 cm pathlength) at 280 nm using a Philips PU8700 Scanning Spectrophotometer. Serum albumin was used as a standard. The total osmotic pressure of the haemolymph was measured on 20 μl subsamples using a Wescor 550 vapour pressure osmometer. The concentrations of organic acids present in the haemolymph were estimated using a high performance liquid-chromatography (HPLC) system (Gilson 715, France) coupled to a Brownlee Polypore H organic acid analysis column (200 x 4.6 mm). Two different techniques were used to extract organic acids from the haemolymph samples; perchloric acid extraction (Gäde *et al.*, 1978) and an H_2SO_4 / diethyl ether procedure (Guerrant *et al.*, 1982). The former method was preferred for consistency of extraction. Extracted samples (20 μl) were introduced into the column using a 50 μl syringe. The column was maintained at 70°C in a Jones chromatography heater and eluted with H_2SO_4 (0.01 M) at a rate of 0.3 ml. min^{-1} . Organic acids, when present, were detected by UV detector at a wavelength of 210 nm. The results were recorded by the software system. The following standards were run: acetate, formate, fumarate, lactate, malate, malonate, oxaloacetate, propionate, pyruvate, succinate.

Construction of non-bicarbonate buffer lines and calculation of pK'_1

Haemolymph for the construction of CO_2 equilibrium curves was obtained by pooling samples left over after the analyses, outlined above, had been carried out. Mixed samples were tonometered, at 10°C , in the BMS II, against a range of CO_2 tensions (0.07 to 0.80 kPa [0.5 to 6.0 Torr]) supplied by precision gas mixing pumps (Wostoff, Bochum, Germany). The samples were tonometered for 15 min to allow complete equilibration to occur before the total CO_2 content of replicate samples of haemolymph was determined using 50 μl aliquots (Cameron, 1971). The pH of the sample was also measured at this time using the method outlined above. Using these data both the concentration of HCO_3^- present in the haemolymph ($[\text{HCO}_3^-]$) and the

functional $pK'1$ *in vitro* were then calculated for oxygenated samples of pooled haemolymph, at different pH values, and at a temperature of 10°C, using the equations

$$(1) [HCO_3^-] = CCO_2 - a.PCO_2$$

and the Henderson-Hasselbalch equation in the form

$$(2) pK'1 = pH - \log ([HCO_3^-] / a.PCO_2)$$

where a is the solubility coefficient of CO_2 in sea water (6.23 mmol l⁻¹ Pa⁻¹ at 10°C-calculated from Harvey, 1955), CCO_2 is the total CO_2 content of the haemolymph and PCO_2 is the partial pressure of CO_2 in the haemolymph. The concentration of CO_3^{2-} was not calculated as it was assumed that within the pH range measured in the haemolymph of *P. maximus*, the actual amount present would be negligible. *In vitro* non-bicarbonate buffer lines for scallop haemolymph were then constructed by plotting $[HCO_3^-]$ against pH.

Calculation of PCO_2 and $[HCO_3^-]$ *in vivo*

Values for *in vivo* PCO_2 and $[HCO_3^-]$ were calculated using measured values of *in vivo* pH and CCO_2 and the Henderson-Hasselbalch equation in the following forms

$$(3) pH = pK'1 + \log (CCO_2 - (a.PCO_2) / (a.PCO_2))$$

and

$$(4) pH = pK'1 + \log ([HCO_3^-] / a.PCO_2)$$

Again it was assumed that the concentration of CO_3^{2-} present would be negligible at physiological pH and so this was ignored.

Statistical analysis of the data

Time course data were analysed using both ANOVAR and 2s 't' test. Differences were considered statistically significant at $P = 0.05$. All values are expressed as means + 1 Standard Deviation.

Results

During emersion there was little activity noticed in the scallops, with the following exception. After removal from sea water, scallops exhibited a typical 'emersion' response which consisted of a series of rapid shell movements or adductions. Such behaviour was evident within 5 minutes of emersion and continued, although with rapidly decreasing frequency, for up to 12 h (Duncan, unpubl. obs.). These valve adductions appear to be identical to those used to propel the animal through the water during escape swimming in response to predators (Thomas and Gruffydd, 1971). Within 72 h most of the emersed scallops were dead or dying, even when relative humidity was maintained close to 100%.

The CO_2 equilibrium curves constructed for scallop haemolymph were not significantly different from those of sea water. The equation to describe the non-bicarbonate buffer line for the haemolymph was $[\text{HCO}_3^-] = 29.67 - 3.88 \text{ pH}$. Air equilibrated scallop haemolymph had a pH of 7.21 ± 0.03 ($n=8$).

The PO_2 of the haemolymph decreased significantly within the first 2 h of emersion from $15.8 \pm 2.1 \text{ kPa}$ (118.7 Torr) to $7.7 \pm 2.0 \text{ kPa}$ (57.5 Torr) (Fig. 1). Thereafter there was no significant change in PO_2 over the next 24 h. By 72 h as most of the animals were dead or dying it became difficult to extract haemolymph values. This was due to the fact that in most cases the heart had stopped beating and the ventricle had collapsed, thus forcing haemolymph from the heart chambers. Consequently we have only one reliable PO_2 reading (3.7 kPa (28.1 Torr)) for this time interval.

The pH of the haemolymph also decreased significantly within the first 2 h from 7.36 ± 0.08 to 7.11 ± 0.07 (Fig. 1). Haemolymph pH continued to decline over the next 24 h ($\text{pH}=6.91 \pm 0.13$) although none of these differences were statistically significant. By 72 h there had been a further significant decrease in pH to 6.68 ± 0.07 ($n=2$).

Accompanying the decline in pH was a progressive, and significant, increase in $[\text{HCO}_3^-]$ throughout the first 8 h of the emersion period, from an initial value of 1.34 ± 0.24 to $3.12 \pm 0.42 \text{ mmol. l}^{-1}$ (Fig. 1). There were no significant changes recorded over the following 16 h although $[\text{HCO}_3^-]$ did seem to decrease slightly (24 h = $2.63 \pm 0.29 \text{ mmol. l}^{-1}$). However, by 72 h emersion $[\text{HCO}_3^-]$ showed a significant increase to 3.37 ± 0.27 ($n=2$).

There was a marked increase in the calculated PCO_2 from 0.13 to 0.77 kPa (1.0 to 5.8 Torr) over the first 8 h emersion (Fig. 2). There was no further change until between 24 and 72 h of emersion when there was a sharp increase in PCO_2 from 0.75 to 1.63 kPa (5.6 to 12.2 Torr).

The acid-base disturbances recorded in scallop haemolymph can be followed more clearly if we present data for pH and HCO_3^- in the form of a Davenport (1974) diagram (Fig. 3). Here we see that for 0-24 hours of emersion a progressive and uncompensated respiratory acidosis has developed. This continued until at 72 h, the time when substantial mortalities begin to occur, there was also some evidence of a small metabolic component to the acidosis, indicated by the downward deviation from the buffer line (Davenport, 1974).

Effect of emersion on osmotic and ionic constituents

There were no significant differences in the osmotic pressure of scallop haemolymph throughout the emersion period, even after 72 h (Table I). This remained constant at 989.2 ± 45.3 mOsm.Kg⁻¹. Nor were there any significant differences noted in the concentration of haemolymph protein throughout the emersion period (Table I). There were, however, significant differences in the concentrations of both of the divalent ions present in the haemolymph during the emersion period (Fig. 4).

During the first 8 h there were no significant changes in the concentration of Ca^{2+} in the haemolymph ($[\text{Ca}^{2+}]$). Between 8 h and 12 h, however, there was a small but significant increase in $[\text{Ca}^{2+}]$ from 11.98 ± 0.94 to 14.56 ± 0.81 mmol.l⁻¹. This increase was sustained over the next 12 h, although by 72 h there was no significant difference between the $[\text{Ca}^{2+}]$ in the haemolymph of experimental and control animals.

Conversely, there was a large and significant decrease in the concentration of Mg^{2+} in the haemolymph ($[\text{Mg}^{2+}]$) from 37.58 ± 6.97 to 26.66 ± 1.14 mmol.l⁻¹ within the first 2 h emersion. Thereafter there was no significant change in the concentration of Mg^{2+} in the haemolymph until after 24 h when a significant increase was recorded returning $[\text{Mg}^{2+}]$ to pre-emersion control values.

Organic acids

The following acids were detected in the haemolymph of *Pecten maximus* during emersion; succinate, lactate, fumarate and propionate. Overall concentrations of these acids were low, and were frequently absent from samples. Acid concentrations were found not to change significantly over the first 24 hours in air (Table 2). By 72 hours, however, all acids had shown substantial increases, although concentrations were still relatively low. Unfortunately, due to limited sample volume, only one 72 hour sample was available for analysis and therefore statistical analysis was not possible. The finding was, however, in keeping with the pattern shown in the Davenport diagram, which also indicated a metabolic component to the acidosis

after this time. Control samples of haemolymph from immersed scallops, taken at 0 and 72 hours, showed no significant differences in acid concentration.

Discussion

During emersion the haemolymph of the scallop *Pecten maximus* was in a state of progressive respiratory acidosis which continued until about 72 h after emersion. After this time the animals began to die. Other marine and fresh water bivalves also experience a respiratory acidosis during emersion except that this is usually partially compensated by an increase in $[\text{HCO}_3^-]$ (Collip, 1920, 1921; Jokumsen & Fyhn, 1982; Byrne & McMahon, 1991; Byrne *et al.*, 1991). Byrne & McMahon (1991) found that even in the fresh water *Anodonta grandis simpsoniana*, which is never normally exposed to air, there was a partially compensated respiratory acidosis and after 96 h emersion, pH stabilised, so that increases in $[\text{HCO}_3^-]$ were not associated with increases in PCO_2 . Such compensation was not evident in the haemolymph of *P. maximus* during emersion. It should also be noted that *A. g. simpsoniana* was able to survive considerably longer periods of emersion than *P. maximus*. Unfortunately, we have no comparable data for the extrapallial fluid of *P. maximus* which would have allowed a comparison with changes in the equivalent fluid in other bivalves (Dugal, 1939; Crenshaw, 1972).

Although there was an increase in $[\text{Ca}^{2+}]$ in *P. maximus* between 8 and 12 h after emersion, the increase (approx. 3 mmol. l^{-1}) was considerably smaller than may have been expected on the basis of previous bivalve studies. Most other authors have noted substantial mobilisation of Ca^{2+} during both hypoxia and emersion, presumably from dissolution of the shell (Collip, 1920, 1921; Crenshaw & Neff, 1969; Crenshaw, 1972; Jokumsen & Fyhn, 1982; Byrne & McMahon, 1991; Byrne *et al.* 1991). To take but one example, in the early work of Collip (1920) on *Mya arenaria*, haemolymph $[\text{Ca}^{2+}]$ was seen to increase at least 6 fold during emersion to a final concentration of 60 mmol. l^{-1} . In fact Byrne & McMahon (1991) (and others) have calculated that there was a direct proportionality between $[\text{Ca}^{2+}]$ and $[\text{HCO}_3^-]$, lending weight to the hypothesis that shell CaCO_3 is used as a buffer in extracellular pH maintenance. Exactly why this mechanism was not found in the scallop *P. maximus* is unknown, but its absence may be correlated with the inability of this animal to withstand prolonged aerial exposure. Other bivalves which are not normally subject to periods of aerial exposure, but which do employ a shell buffering mechanism, can still survive for many days in air and show a rapid return to 'normal' conditions upon re-immersion (Byrne & McMahon, 1991).

The dramatic and sustained decrease in $[\text{Mg}^{2+}]$ of the haemolymph of *P. maximus* is also problematical. It might have been expected for $[\text{Mg}^{2+}]$ of the haemolymph to increase, perhaps due to the dissolution of any carbonates of magnesium present in the shell. Unfortunately we do

not have much comparable information for emersion related changes in $[Mg^{2+}]$ of haemolymph from other species and such data that we have obtained has indicated that the $[Mg^{2+}]$ of the haemolymph increased upon emersion (for example see Spicer *et al.* 1988). So there remains the possibility of an active process removing Mg^{2+} ions from the extracellular fluid. As a possible line of further enquiry, it is suggested that Mg^{2+} movements were in some way linked to either changes in intracellular ATP and/or the activity of this animal. This may at first sound implausible in the case of an emersed scallop, except that within the first few hours of emersion there are rapid closures of the shell, identical to those used to propel the animal when it is immersed. Recently a strong link has been established between $[Mg^{2+}]$ of the haemolymph and activity in a high shore amphipod; the more active the amphipod, the less Mg^{2+} is present in the haemolymph (Spicer *et al.*, submitted). It is suggested that the scallop is physiologically 'ready' if favourable conditions return (immersion) to remove itself from an unfavourable environment. It would be interesting to record Mg^{2+} fluxes in the extracellular fluids of active and passive *P. maximus* and determine if $[Mg^{2+}]$ can be correlated with activity in this species.

Acidosis in emersed bivalves very often has a metabolic component. In the clam *Rangia cuneata*, which closes its valves when out of water, there was a substantial reduction in haemolymph pH associated with the production of metabolic end products (Fyhn & Costlow, 1975). In bivalves which gape during emersion the primarily respiratory acidosis incurred is often compounded by the production of organic end products such as succinic and lactic acids (Dugal, 1939; Crenshaw & Neff, 1969). This also appears to be the case in *P. maximus* during emersion, although only after 24 hours in air since no significant changes in the detected acids were apparent before this time. The actual concentrations of acids detected were low, with a maximum value for any acid of 3 mmol. l^{-1} (72 hour succinate). Comparison with the crustacean species *Nephrops norvegicus* (Spicer *et al.*, 1990) and the echinoderm, *Echinus esculentus* (Spicer *et al.*, 1988) under similar experimental emersions suggests that *Pecten maximus* has a relatively low anaerobic capacity. For example, values for lactate in the former species were 9.43 and 0.167 mmol. l^{-1} respectively after 12 hours, but only 0.22 mmol. l^{-1} in the scallop. It is of interest, in this connection, that an examination of the Davenport diagram, presented in Fig. 3, also seems to confirm this finding of a relatively low anaerobic metabolism, since there appears to be only a small metabolic acid contribution to the overall acidosis despite the recorded adduction activity during emersion. The observed acidosis was therefore almost exclusively respiratory in origin.

In conclusion we can say that the acid-base disturbance during emersion in the scallop *P. maximus*, although it had some features in common with what is known for other bivalves (i.e. presence of respiratory acidosis), was distinctive to that species. The pattern of acid-base disturbance was even different from other bivalves which are similar in that they are never subject to periods of aerial exposure. The haemolymph of *P. maximus* showed a progressive

respiratory acidosis during emersion that was neither ameliorated by the 'release' of shell buffering substances nor severely compounded by the production of organic acids. The fact that death when emersed came more rapidly than has been recorded even in other fully aquatic bivalves, only serves to illustrate the seriousness of the acid-base disturbance incurred and the state of compromise that existed for the exchange of respiratory gases. If organic acids are not produced in large quantities by scallops during emersion this may have implications for the quality of 'live' scallop meat post harvest. This would be quite different from the situation in *Nephrops norvegicus*, where post harvest emersion of intact animals results in substantial accumulation of lactic acid in the haemolymph (Spicer *et al.* 1990).

Acknowledgements

This study was part of a wider project on the post-harvest physiology of *Pecten maximus* sponsored by grants from Highland Enterprise and the Worshipful Company of Fishmongers.

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Table 1 The osmotic pressure and protein content of the haemolymph of *P. maximus* during emersion at 10°C. Values given are means (+ S.D.), n = 5 except 72 h where n=1.

Time (h)	Osmotic pressure (mosm.kg ⁻¹)	Protein (mg.ml ⁻¹)
0	989.2 (45.3)	0.43 (0.17)
2	982.6 (7.7)	0.37 (0.11)
4	1012.2 (47.0)	0.48 (0.20)
8	965.4 (47.7)	0.32 (0.11)
12	1005.6 (50.6)	0.33 (0.15)
24	967.8 (24.7)	0.39 (0.12)
72	1001.0	0.44
72 control	988.0 (29.4)	0.40 (0.13)

Table 2 Organic acid concentration changes in the haemolymph of *P. maximus* during emersion at 10°C. Values given are means (+S.D), n=5 except 72 h where n=1.

Emersion time (hours)	Haemolymph acid concentration mmol. l ⁻¹			
	Succinate	Lactate	Fumarate	Propionate
0	0.52 (0.68)	0.13 (0.30)	0.0005 (0.0007)	0.108 (0.11)
2	0.35 (0.48)	0.02 (0.03)	0.0003 (0.0007)	0.005 (0.01)
4	1.67 (0.65)	0.15 (0.21)	0.0053 (0.0084)	0.107 (0.11)
8	0.86 (0.83)	0.08 (0.06)	0.0010 (0.0010)	0.110 (0.11)
12	0.94 (1.03)	0.22 (0.30)	0.0031 (0.0052)	0.080 (0.11)
24	0.37 (0.51)	0.15 (0.28)	0.0008 (0.0011)	0.061 (0.08)
72	3.0	1.62	0.0170	1.350
72 control	0.14 (0.27)	0.06 (0.13)	0.0017 (0.0011)	0.030 (0.06)

Figure 1. Changes in PO_2 , pH and bicarbonate ($[\text{HCO}_3^-]$) in the haemolymph of the scallop *Pecten maximus* (10°C) during experimental emersion. Each point is the mean of 5 determinations + S.D except PO_2 after 72 h where $n = 1$

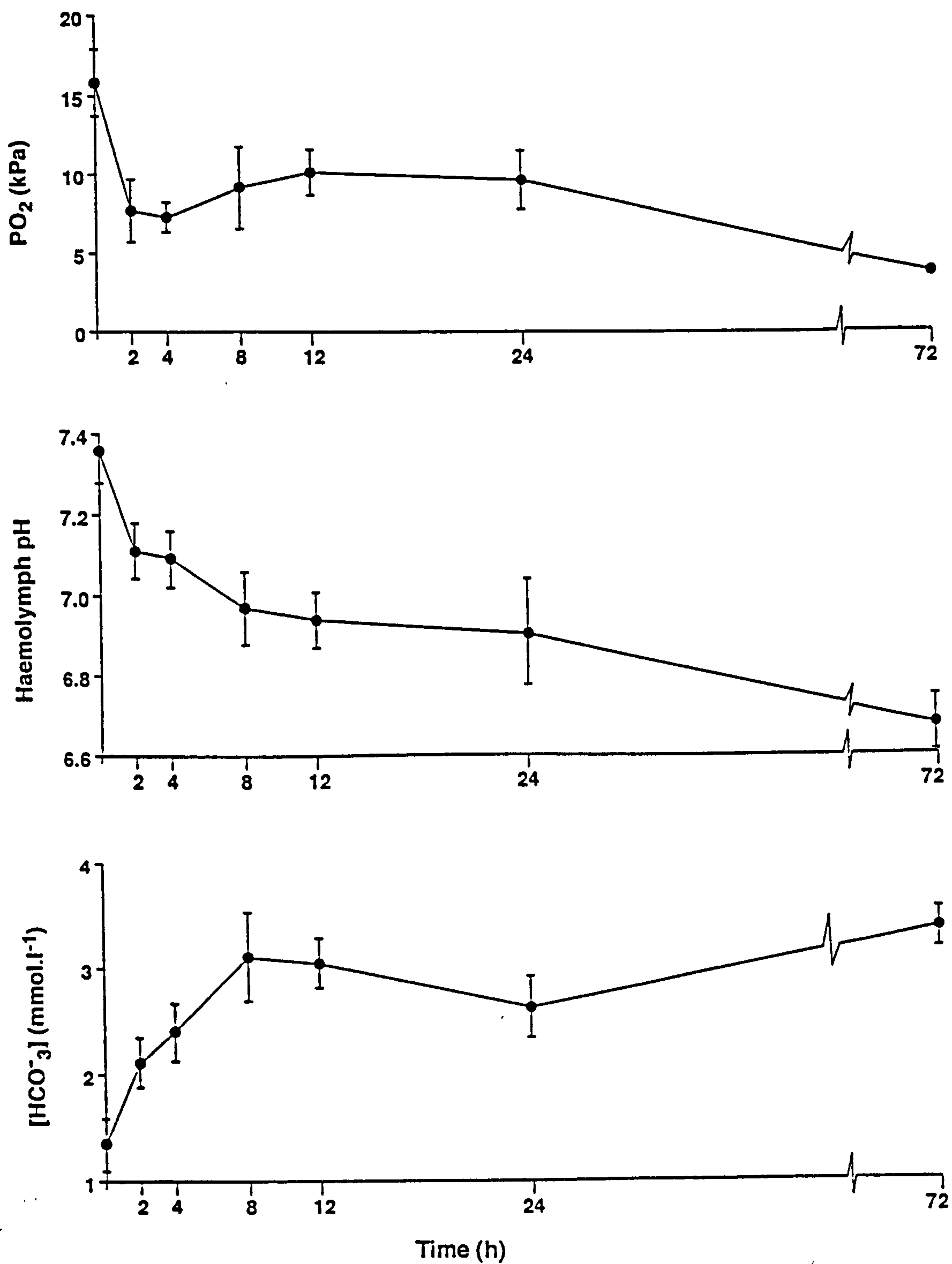


Figure 2. Changes in the calculated PCO_2 in the haemolymph of the scallop *Pecten maximus* during experimental emersion at 10°C .

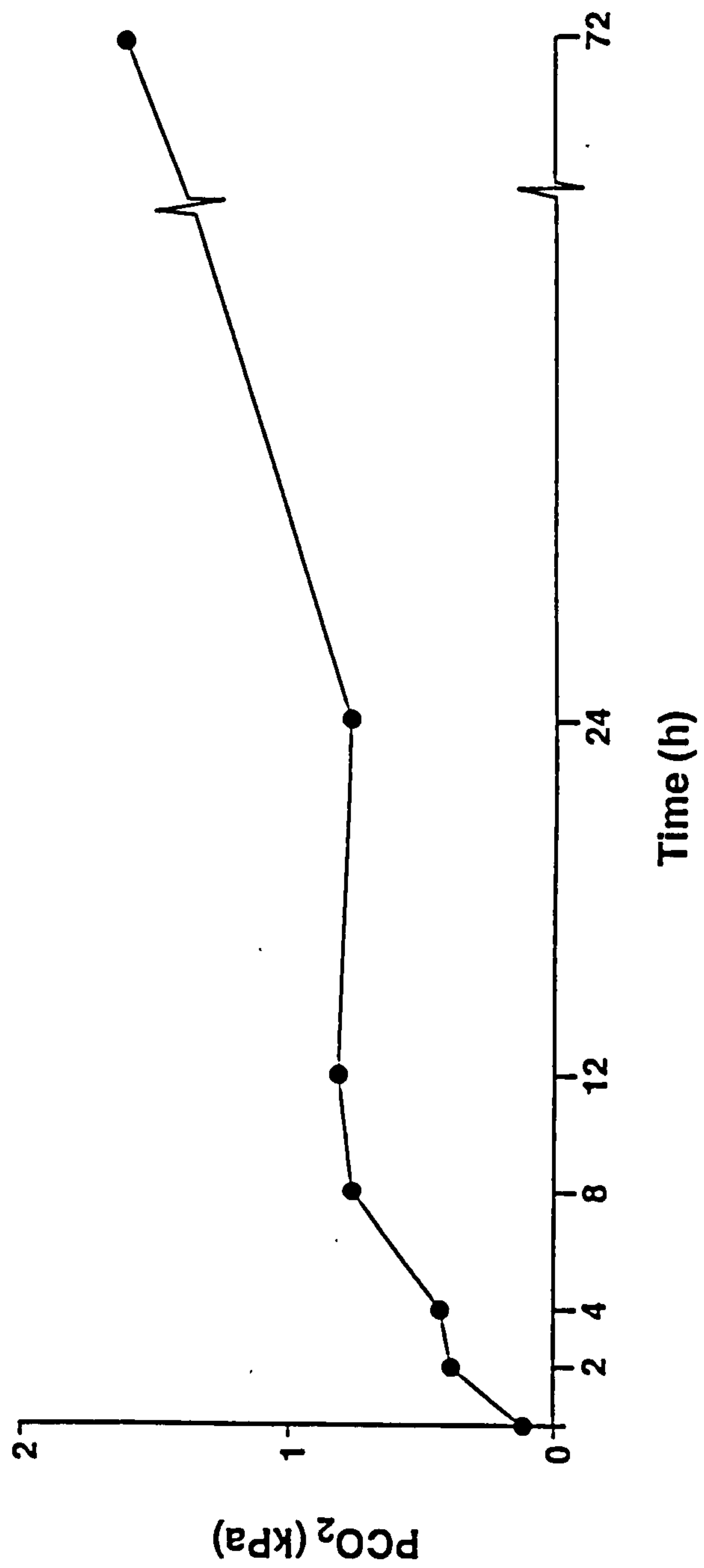


Figure 3. Graphic representation (after Davenport, 1974) of relationship between concentration of bicarbonate ions ($[\text{HCO}_3^-]$) and pH present in the haemolymph of *Pecten maximus* under conditions of emersion (10°C). Numbers beside data points represent duration of exposure (h). Data points are given + S.D. Oblique line represents mean non-bicarbonate buffer line for oxygenated haemolymph at 10°C .

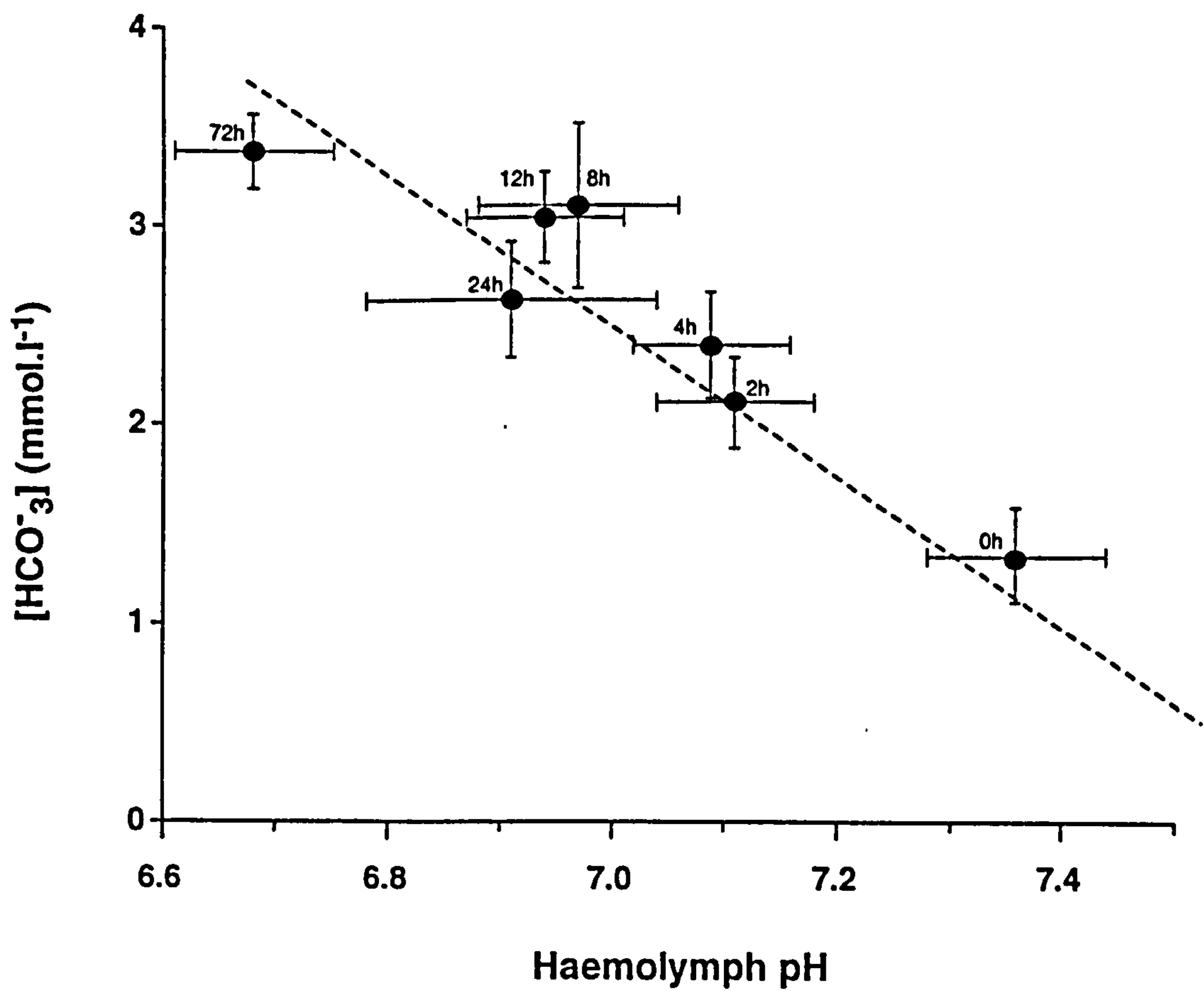


Figure 4. Changes in the concentrations of the divalent ions, Ca^{2+} (open symbols) and Mg^{2+} (closed symbols) in the haemolymph of the scallop *Pecten maximus* (10°C) during experimental emersion. Each point is the mean of 5 determinations + S.D.

